IDENTIFICATION OF NEW MOLECULAR TARGETS FOR THE TREATMENT OF NEUROBLASTOMA AND MEDULLOBLASTOMA

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Zurich, 2012
Fabiana Salm

To my mother
Beita

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ABBREVIATIONS

4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
AKT	v-akt murine thymoma viral oncogene homolog
ALK	anaplastic lymphoma kinase
APC	adenomatous polyposis complex
BAD	BCL2-associated death promoter
BCL2	B-cell leukemia/lymphoma 2
BCLXL	B-cell leukemia/lymphoma extra large
BIRC5	survivin
CCND1	Cyclin D
CNS	central nervous system
D/N	desmoplastic medulloblastoma
DAG	diacylglycerol
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FKHR	forkhead box O1
FRS2	fibroblast growth factor receptor substrate 2

Gab-1	growth factor receptor bound 2 associated protein 1
Grb2	growth factor receptor bound 2
GSK3	glycogen synthase kinase
GWAS	genome wide association studies
IGF-1R	insulin-like growth factor 1 receptor
INRG	International Neuroblastoma Risk Group
IP_3	inositol-1,4,5-triphosphate
IRS1	Insulin receptor substrate 1
LC/A	large cell anaplastic medulloblastoma
LIFR	leukemia inhibitory factor receptor alpha
LOH	loss of heterozygosity
MAPK	mitogen activated protein kinase
MB	medulloblastoma
MBEN	medulloblastoma with extensive nodularity
mTOR	mammalian target of rapamycin
NB	Neuroblastoma
NME1	nucleoside diphosphate kinase A
OSM	oncostatin M
PDGFR	platelet-derived growth factor receptor
PDK1	3-phosphoinositide-dependent protein kinase 1
PHOX2B	paired-like homebox 2B
PI	phosphatidylinositol
PI3K	phosphoinositide 3-kinase, phosphatidylinositol 3-kinase, PI 3-kinase
PI(5)P	phosphatidylinositol-5-monophosphate
$PI(4,5)P_2$	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PLCγ	phospholipase C γ
PTCH1	protein patched homolog 1
PTB	phosphotyrosine binding

RSK2	ribosomal protein S6 kinase 2
RTK	receptor tyrosine kinase
SH2	Src homology 2
Shh	sonic hedgehog
siRNA	small-interfering RNA
SMO	smoothened protein
SNP	single nucleotide polymorphism
SOS	son of sevenless
STAT3	signal transducer and activator of transcription 3
SUFU	protein suppressor of Fused
TK	tyrosine kinase
VEGFR	vascular endothelial growth factor receptor
WHO	world health organization
Wnt	wingless

SUMMARY

Embryonal tumors represent an important group of childhood malignancies. Among them, medulloblastoma (MB) is a tumor of the central nervous system that arises in the cerebellum, while neuroblastoma (NB) is an extra cranial solid tumor that originates from the sympathetic nervous system. Despite intense multimodality therapy, the survival rates for high-risk patients remain very little. Therefore, novel therapeutic strategies are urgently required.

Kinases have drawn much of the attention for the design of new targeted therapies due to their role in oncogenic transformation. In the present thesis, we used a loss-of-function kinome-wide RNA interference (RNAi) screen aimed at identifying new kinases which are critical for promoting drug resistance, a major cause of treatment failure in neuroblastoma. Several kinases have been identified amongst which the fibroblast growth factor receptor 2 (FGFR2) represented the most attractive candidate for further validation studies. RNAi mediated silencing of FGFR2, as well as pharmacological inhibition of the kinase activity of FGFR2, sensitized NB cells to cisplatin, a commonly used chemotherapy for the treatment of neuroblastoma. Analysis of the expression of FGFR2 in primary NB revealed that FGFR2, as well as its ligand FGF-2 are consistently expressed, indicating the presence of

an autocrine loop. Moreover, a cDNA microarray analysis comprising data of over 250 NB samples revealed that FGFR2 over-expression correlates with *MYCN* amplification and advanced stage disease, demonstrating for the first time the clinical relevance of FGFR2 in neuroblastoma.

Receptor tyrosine kinases (RTK) and their downstream signalling cascades have been shown to play a crucial role in cancer development. In neuroblastoma and medulloblastoma, the phosphoinositide 3-kinase (PI3K) has been found to have an important role in mediating pro-survival signals from different growth factor receptors such as IGF-1R or ERBB2. In a further project, we have analyzed the therapeutic potential of targeting the IGF-1R/PI3K axis in MB and NB with R1507a humanized monoclonal antibody against the IGF-1R, and PIK75 class I_A PI3K inhibitor. Our findings demonstrate that a subset of NB and MB cell lines were responsive to these agents, particularly when combined with conventional chemotherapies. However, other agents targeting different RTKs or PI3K have previously demonstrated to have anti-proliferative effects in preclinical models, but translating the results into a clinical setting has been rather challenging. A better knowledge of the mechanisms of action of the signalling components downstream of RTKs may contribute to the identification of novel and suitable therapeutic strategies.

In order to gain further insights into the mechanisms underlying the role of the different PI3K isoforms in MB in cell survival, we analyzed the impact of RNAi-mediated down-regulation of p110α and p110δ on the global gene expression of MB cells by means of DNA microarrays. By using bioinformatic tools, a comparative analysis of the sets of genes, which were differentially expressed, revealed c-Myc as the transcription factor, whose network of genes was mostly deregulated. This transcriptional network included the α-subunit of the receptor for the leukemia inhibitory factor (LIFRα) as target gene, which was one of the most significantly deregulated genes upon the downregulation of *PIK3CA,* encoding for the isoform p110α. LIFRα expression was found

elevated in primary MB, in comparison to normal cerebellum. Targeting the LIFRα by RNAi or by a neutralizing antibody significantly impaired MB cell proliferation. Being a downstream effector of the well characterized oncogenes *PIK3CA* and *MYC* in MB, LIFR represents an interesting target gene for further analysis.

All together, the results obtained in this thesis provide insights into the role of kinase signalling in neuroblastoma and medulloblastoma. Further validations and clinical-oriented studies will hopefully contribute to the improvement of the therapeutic strategies for the treatment of these young patients.

ZUSAMMENFASSUNG

Embryonale Tumoren entstehen durch Entartung unreifer Zellen während der embryonalen Organ- und Gewebeentwicklung. Zu diesen zählen Medulloblastome (MB), bösartige Tumore des zentralen Nervensystems, die im Kleinhirn entstehen, und Neuroblastome (NB), extrakranielle solide Tumore des unreifen sympathischen Nervensystems. Die Behandlung solcher Tumore besteht aus der operativen Tumorentfernung und einer Kombination aus intensiver Chemo- und Strahlentherapie. Trotz dieser aggressiven therapeutischen Ansätze sind die Überlebensraten für Hochrisiko-Patienten nur sehr gering. Neue therapeutische Strategien sind daher dringend erforderlich.

Proteinkinasen stehen im Forschungsinteresse für die Entwicklung neuartiger zielgerichteter Krebstherapien, da sie von zahlreichen genetischen Veränderungen bei der Entstehung verschiedener Krebsarten betroffen sind. Das Hauptziel der vorliegenden Dissertation lag in der Identifizierung neuer Protein- und Lipidkinasen, die für die Regulierung der Resistenzmechanismen gegenüber Chemotherapeutika zuständig sind. Chemoresistenz stellt eine der Hauptursachen für Therapieversagen in NB Patienten dar. Daher wurde ein RNA Interferenz (RNAi) Screen in Kombination mit Cisplatin, eines der

meistverwendeten Chemotherapeutika, durchgeführt. Somit war eine systematische Untersuchung aller Kinasen des menschlichen Genoms möglich, die aufgrund der Herunterregulierung ihrer Aktivität die Empfindlichkeit zu Cisplatin erhöht haben. Mehrere Kinasen wurden dadurch identifiziert, darunter der Rezeptor für Fibroblasten-Wachstumsfaktoren FGFR2. Um die klinische Relevanz von FGFR2 zu validieren, wurde die Expression von FGFR2 und dessen Ligand FGF-2 in primären NB Tumoren analysiert. Sowohl FGFR2 als auch FGF-2 wurden detektiert, was auf die Anwesenheit einer autokrinen Schleife hinweist. Des Weiteren zeigten DNA Microarray Analysen mit Daten von über 250 Neuroblastom Patienten, dass die Expression von FGFR2 mit einer *MYCN*-Amplifikation korreliert, einem Tumormarker schlechter Prognose. Schliesslich konnte gezeigt werden, dass die Hemmung von FGFR2 mit einem niedermolekularen Inhibitor die Sensitivität gegenüber Cisplatin in NB Zellen erhöht, was auf seine mögliche Verwendung als Angriffspunkt für eine medikamentöse Tumortherapie hindeutet.

Rezeptor-Tyrosin-Kinasen (RTK) und die von ihnen regulierten Signalkaskaden spielen eine entscheidende Rolle bei der Krebsentstehung. Phosphoinositid-3-Kinasen (PI3K) werden dabei als wichtige Vermittler von Wachstumssignalen, die aus verschiedenen Rezeptoren stammen, bezeichnet. In einem weiteren Projekt wurden verschiedene Ansätze verwendet, um das therapeutische Potenzial der IGF-1R/PI3K-Achse in NB und MB zu analysieren. Daher wurde das antiproliferative Potenzial von R1507, ein monoklonaler Antikörper gegen IGF-1R, und PIK75, ein isoform-selektiver Inhibitor der Klasse I_A PI3K in verschiedene NB und MB Zelllinien getestet. Beide Wirkstoffe zeigten antiproliferative Effekte in einem Teil der Zelllinien. Zusätzlich wurden diese Effekte in Anwesenheit von Chemotherapeutika verstärkt. In ähnlicher Weise haben auch andere Studien nahe gelegt, dass die Effektivität einzelner Inhibitoren durch Kombination mit anderen Inhibitoren oder Chemotherapeutika deutlich gesteigert werden kann. Eine bessere Kenntnis über die

Wirkmechanismen von PI3K wird daher zur Entwicklung geeigneter Kombinationen beitragen.

Ein weiteres Projekt dieser Dissertation war daher die Identifizierung MB-spezifischer PI3K-regulierter Gene. Zu diesem Zweck wurden MB Zellen, in denen die verschiedenen PI3K Isoformen *PIK3CA* und *PIK3CD* herunterreguliert waren, einer cDNA Microarray Analyse unterzogen, um ihre Expressionsprofile zu analysieren. Dieser Versuch ergab eine Liste von Genen, die spezifisch durch p110α, die vom *PIK3CA* kodiert wird oder p110δ, die vom *PIK3CD* kodiert wird, reguliert wurden. Mithilfe einer bioinformatischen Analyse war die Identifizierung deregulierter, transkriptioneller Netzwerke möglich. Daraus ergab sich c-Myc als der Transkriptionsfaktor, dessen Gene am signifikantesten differentiell deprimiert wurden. Unter diesen befand sich auch der LIF Rezeptor (LIFRα). Im Vergleich zu normalem Kleinhirn zeigten MB Zellen eine erhöhte LIFR Expression. Um die funktionelle Relevanz von LIFR im Medulloblastoma zu beurteilen, wurde seine Funktion mithilfe von siRNAs herunterreguliert oder durch einen Antikörper gehemmt. Dies führte zu einer verringerten Zellproliferation. Da LIFR unter der Regulierung bekannter Onkogene (*PIK3CA* und *MYC)* liegt, stellt es einen interessanten Kandidaten für weitere Studien dar.

Zusammengefasst trägt die vorliegende Doktorarbeit zu einem besseren Verständnis über die Rolle von Kinasen im Signalsystem von Neuroblastom und Medulloblastom bei. Weitere Validierungen sowie klinisch-orientierte Studien werden hoffentlich zu einer verbesserten Behandlung von Patienten mit diesen Krebsarten beitragen.

CHAPTER 1

INTRODUCTION

1.1 Neuroblastoma

Neuroblastoma is an embryonal tumor that arises from the developing neural crest tissue [1]. Tumors can therefore develop anywhere in the sympathetic system, most of them arising in the adrenal medulla. Other common sites of the disease are neck, chest, abdomen and pelvis [2]. Neuroblastoma is the most frequently diagnosed cancer during the first year of life, the median age at the time of the diagnosis being 17 months [3, 4]. It accounts for 7-10 % of all cancers in children younger than 15 years and around 15% of all childhood cancer related mortality [5].

Neuroblastomas exhibit very heterogeneous clinical behaviors. On the one hand, a subset of tumors undergo spontaneous and complete regression of the tumor with little or no therapy, while other tumors show rapid progression and are resistant to intensive multimodality therapies [2]. Approximately half of all cases are classified as high-risk for disease relapse at the time of diagnosis. Although the outcomes for patients with neuroblastoma have substantially improved in the past few decades, this improvement has mainly benefited patients with the more benign form of the disease. The overall survival rate for high-risk neuroblastoma patients is still less than 40% [6, 7]. Many efforts have been done in the past years,

and are still ongoing, to understand the biology of neuroblastoma and to better stratify patients into risk groups. This will definitely help to develop more specific therapies for patient subgroups and hopefully improve the outcomes for neuroblastoma patients.

1.1.1 Genetic alterations

Neuroblastoma predisposition

Recent studies utilizing single nucleotide polymorphism (SNP) arrays of a large number of neuroblastoma patients have made genome-wide association studies (GWAS) possible, which has led to the identification of multiple common genetic variations that predispose to familial or sporadic neuroblastoma.

Familial neuroblastoma is extremely rare, it accounts for only 1-2% of neuroblastoma cases [8-10]. The first identified predisposition gene in familial neuroblastoma was the paired-like homebox 2B gene (*PHOX2B*), which encodes for a transcription factor that is essential in the regulation of neurogenesis [11, 12]. Loss-of-function mutations in *PHOX2B* were, in most of the cases, associated with abnormalities of neural crest derived tissues [11, 12]. Germline mutations in the anaplastic lymphoma kinase (*ALK*) gene were subsequently identified in most of the hereditary neuroblastoma [13]. These mutations are found within the coding exons of its tyrosine kinase domain and result in constitutive activation of the *ALK* oncogene. *ALK* mutations are also found in 5 to 15% of somatically acquired neuroblastomas, underlying its importance for the development of neuroblastoma. Mutations in *ALK* and *PHOX2B* account for the majority of hereditary cases, but additional genes may still be discovered.

In sporadic neuroblastoma cases, three common alleles with risk polymorphism have been identified, which are associated with high-risk neuroblastomas. These common SNP variations are found within the genes *FLJ22536* at chromosome band 6p22.3, *BARD1* at 2q35 and *LMO1* at 11p15 [14-17]. The identification of these SNP predisposition loci

marks areas of the genome in which somatically acquired variations contribute to disease progression.

Somatically acquired genetic variations

Chromosomal aberrations in neuroblastoma include loss or gain of genetical material. One of the first genetic aberrations found in neuroblastoma that could be consistently used as a biomarker for clinical phenotyping is genomic amplification of *MYCN* at chromosome band 2p24 [18-20]. Approximately 20% of primary tumors display amplification of *MYCN*, which correlates with advanced stage disease and with adverse patient outcome [21]. Other DNA copy number aberrations have a prognostic value as well. Loss of heterozygosity (LOH) at chromosome 1p is identified in about one third of neuroblastomas and is highly associated with *MYCN* amplification and advanced disease stage [22-24]. There is evidence that allelic loss of 1p36 predicts an increased risk of relapse in patients with localized tumors [25, 26]. Despite intensive investigation, there is little known about tumor suppressor genes at 1p36 that are important for neuroblastoma pathogenesis. Allelic loss at chromosome 11q, most frequently found at 11q23, is present in 35-45% of primary tumors [27]. It is rarely seen in tumors with *MYCN* amplification, but correlates with other high-risk features, such as advanced stage, unfavorable histology and older age [27, 28]. The most frequent allelic gain is at chromosome 17q, which is identified in over 50% of primary tumors [29, 30]. It results from unbalanced translocations and is associated with decreased survival [30, 31]. Breakpoints vary significantly, but the most commonly affected region is at 17q22. Candidate genes overexpressed as a consequence of 17q gain include *BIRC5* (survivin), *NME1* and *PPMID* [32-34]. The exact role of these genes in neuroblastoma oncogenesis remains yet to be studied.

The total DNA content (ploidy) is considered to be a valuable prognostic factor as well and is frequently used for patient risk stratification. DNA copy-number aberrations are classified into two categories: whole-chromosome gains or segmental chromosomal aberrations [2, 35]. Tumors with whole chromosome gains or losses display hyperdiploidy are

associated with lower risk disease and favorable outcomes. Segmental chromosomal aberrations are associated with high-risk disease and worse outcomes. A whole genome copy number analysis that included about 500 neuroblastoma samples demonstrated that the strongest predictor of relapse was the presence of segmental chromosomal alterations, independently of other prognostic factors including age, stage, and *MYCN* status [35].

1.1.2 Patient subgroups and therapeutical approaches

Due to the biological heterogeneity of the tumors in newly diagnosed neuroblastomas, it has been difficult to develop a risk-stratification system that classifies patient subgroups. In an effort to improve the classification scheme, a large international consortium analyzed data of 8800 patients between 1990 and 2002 and proposed the new International Neuroblastoma Risk Group (INRG) classification system [36]. This system classifies patient groups based on the assessment of 13 prognostic factors, including age at diagnosis, INRG tumor stage histologic category, grade of tumor differentiation, DNA ploidy and copy number status at the *MYCN* oncogene locus and at chromosome 11q. Four clinical risk groups with different survival rates have been proposed: high-risk-, intermediate-risk-, low-risk patients and patients with tumor stage 4S. An overview of the different risk categories is presented in Table 1-1.

The advances made by the INRG committee have facilitated the selection of treatment regimens for neuroblastoma patients. Despite the improved risk-based therapies, there is still an ongoing interest in discovering new tumor markers with prognostic value to more precisely assign patients to appropriate therapy. Especially in the case of high-risk patients, for which, as described below, the survival rate remains dismal.

Variable	Prognostic Category			
	Low Risk	Intermediate Risk	High Risk	Tumor Stage 4S
Pattern of disease	Localized tumor	Localized tumor with locoregional lymph-node extension; metastases to bone marrow and bone in infants	Metastases to bone marrow and bone (except in infants)	Metastases to liver and skin (with minimal bone marrow involvement) in infants
Tumor genomics	Whole-chromosome gains	Whole-chromosome gains	Segmental chromosomal aberrations	Whole-chromosome gains
Treatment	Surgery	Moderate-intensity chemotherapy; surgery	Dose-intensive chemotherapy, surgery, and external beam radiotherapy to primary tumor and resistant metastatic sites; myeloablative chemotherapy with autologous hematopoietic stem-cell rescue; isotretinoin with anti-GD2 immunotherapy	Supportive care
Survival rate	> 98 %	90-95 %	40 to 50 %	> 90 %

Table 1-1. Patient stratification and risk adapted treatments (adapted from [21]).

Low-risk patients

Low-risk patients are those younger than one year at the time of diagnosis. They present with localized tumors, with whole chromosome gains and lack *MYCN* amplification [36]. The treatment of these patients usually consists of surgical tumor resection and, only in some cases, is followed by low dose chemotherapy (carboplatin, doxorubicin or etoposide) [21, 37]. This patient group has an excellent outcome with a disease-free survival rate > 98% [21]. Stage 4S disease is considered a low-risk group, because of its unique favorable biological feature and its high incidence of tumor regression. The treatment is highly dependent on the clinical

presentation. In most of the cases, only supportive care is required. The disease free survival rate for patients with 4S tumors is > 90% [38, 39].

Intermediate-risk patients

Intermediate-risk patients are those generally older than one year at the time of diagnosis and present with tumors spreading to locoregional lymph nodes or bone marrow. Genetic alterations in these tumors are often whole chromosomal gains, which are associated with a more favorable outcome. For this patient group, treatment includes surgery followed by subsequent moderate chemotherapy. The outcome for intermediate risk-patients ranges between 90 to 95% [21].

High risk patients

Patients older than one year and presenting with tumors that harbor segmental chromosomal aberrations have an unfavorable outcome are, therefore, considered high-risk patients. Current treatments for high-risk neuroblastomas include intensive chemotherapy cycles prior to surgery. It has been shown that increasing doses of induction chemotherapy are associated with improved response rates [40]. The most commonly used regimen consists of dose-intensive cycles of cisplatin and etoposide alternating with vincristine, doxorubicin, and cyclophosphamide [41].

Myeloablative chemotherapy administered after the induction therapy and followed by autologous stem cell transplantation has been shown to improve disease-free survival rates [7, 42] and is considered by many groups to be part of the standard treatment of high-risk neuroblastoma [21]. Following myeloablation, patients are frequently treated with 13-cis-retinoic acid in order to induce terminal differentiation of neuroblastoma and to eliminate residual cancer cells. Although most high-risk tumors initially respond to the therapy, there is a high incidence of relapse (50 - 60%) and for this patient group there is no treatment known to be curative. The presence of residual cancer cells after chemotherapy that display acquired drug resistance, represent the major obstacle for successful treatment in this context.

1.2 Medulloblastoma

Medulloblastoma is an embryonal tumor which arises from immature neuronal precursors in the cerebellum [43]. It is the most common brain tumor in children with an incidence peak at 8 years of age. It is considered as a grade IV tumor by the WHO (World Health Organization) due to its malignant phenotype. [44]. Medulloblastoma patients can be characterized as high-risk and standard-risk patients based on the clinical criteria and tumor histology, as described below and summarized in Table 1-2. Survival for children with medulloblastoma varies according to these parameters. However, brain tumors generally show worse prognosis compared to other pediatric malignancies, and, therefore, account for over 25% of all cancer-related deaths in childhood [45].

1.2.1 Histopathological classification

Medulloblastomas are classified based on their histological features and four variants are recognized: classical medulloblastoma, desmoplastic medulloblastoma (D/N), medulloblastoma with extensive nodularity (MBEN) and large-cell anaplastic medulloblastoma (LC/A). Two further subtypes, melanotic medulloblastoma and medullomyoblastoma, are rarely seen and because of their similar clinical and genetic features to the other medulloblastoma classes, they are often classified as those [44]. Classical medulloblastoma accounts for 73% of all medulloblastomas, while large-cell anaplastic medulloblastoma makes up about 17% and the desmoplastic subtype about 10%, respectively [46]. MBEN and D/N in general have a better outcome than classical medulloblastoma, while large-cell anaplastic medulloblastoma is associated with poor prognosis and short survival. Despite the fact that many medulloblastomas show identical histologic features, they have different responses to the assigned therapies and thus different outcomes. Recent advances in the molecular biology of medulloblastoma have shown the importance of a more accurate classification of patient groups, which includes the genetic profiles of the tumors [47].

1.2.2 Genetic alterations and aberrant signalling pathways in medulloblastoma

The most frequently altered signalling pathways in medulloblastoma are the Sonic hedgehog (Shh) and the Wingless (Wnt) pathway (Figure 1-1). They were first identified from associations with the Gorlin's and the Turcot's syndrome. A recent genome-wide sequencing study in medulloblastoma identified a significant number of somatic mutations in both pathways, confirming their importance for medulloblastoma development [48].

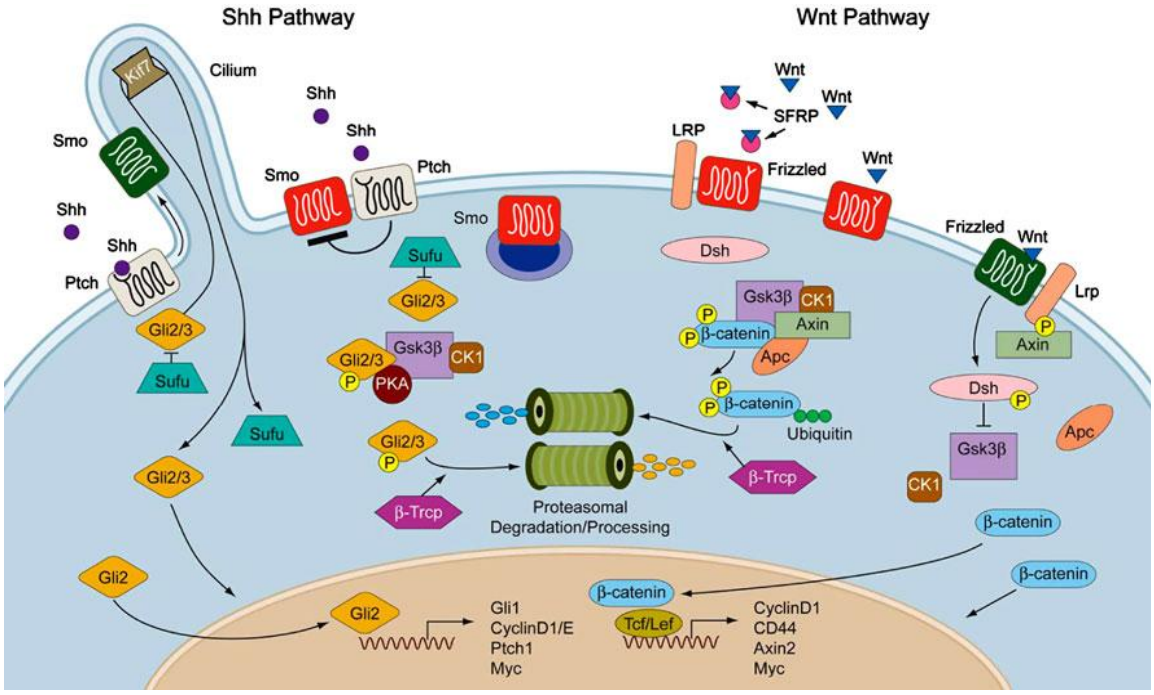

Figure 1-1 Shh and Wnt pathways, from Ellison et al. 2011 [46].

Sonic Hedgehog (SHH) signalling pathway

The Gorlin's syndrome is a rare autosomal dominant disorder associated with skeletal deformities, which shows a high incidence of sporadic medulloblastoma [49]. The disease is characterized by germ-line mutations in the *PTCH1* gene which encodes for a transmembrane receptor that binds to the Hedgehog (HH) family proteins. During the

normal cerebellum development, the receptor Ptch is responsible for binding and repressing the activity of the smoothened protein (SMO) [50]. Once SMO binds to SHH, Ptch-mediated repression is alleviated and the sonic hedgehog (SHH) signalling pathway is activated, which finally triggers the proliferation of granule cells. Loss of function mutations in the *PTCH1* gene may result in aberrant activation of the pathway and deregulated proliferation of granule cells, which might promote malignant transformation [51]. In medulloblastoma, 17% of tumors show mutations in the *PTCH1* gene [48]. In addition to the *PTCH1* mutations, further mutations in the SHH pathway were found in *SUFU* and *SMOH* [46]. About 25% of all medulloblastomas contain mutations in the SHH pathway, most of them have a desmoplastic phenotype (D/N or MBEN), but some large cell anaplastic tumors (LC/A) may also have an SHH profile.

Wingless (Wnt) signalling pathway

A similar study involving the Turcot syndrome identified molecular determinants in the Wingless pathway that are associated with the disease. The Turcot syndrome is a rare heritable disorder, which is characterized by the development of CNS tumors, in particular medulloblastoma and glioblastoma [52]. The Wingless signalling pathway coordinates several developmental processes including the proliferation and fate of neural progenitor cells [53]. The Wnt cascade is activated through the activation of the Frizzled receptor by Wnt ligands. The activation of Frizzled induces the inactivation of the protein complex between APC, GSK3 and Axin, which is responsible for the phosphorylation and degradation of β-catenin. Thus, Wnt pathway activation promotes the translocation of β-catenin to the nucleus and the initiation of the transcription of genes important for the cell cycle, proliferation and differentiation, such as *MYC*, *CCND1* and *NRSF/REST*. Germ-line mutations in the *APC* gene have been first identified and associated with an inherited predisposition to medulloblastoma [52]. Nevertheless, 13-15% of sporadic medulloblastomas have mutations in the Wnt pathway [48, 54]. Most of the mutations reported target the serine 33 and 37 residues of β-catenin

and prevent its phosphorylation and degradation by GSK-3. As a consequence, β-catenin acquires a constitutively active state, which may lead to the development of a malignant phenotype [55]. Wnt tumors have a classical pathology nearly in all cases. They are rarely found in the LC/A subgroup and they have never been reported in the D/N subgroup.

Chromosomal abnormalities

The most frequent chromosomal aberration found in 70% of medulloblastomas is the presence of isochrome i (17q), in 40%-60% of the cases accompanied by loss of 17p [56]. Other chromosomal abnormalities that have been identified are loss of chromosome 10q, 7q and 16q. Gene amplification in *MYC* or *NMYC* has been reported in 4% - 8% of medulloblastomas [57] and is associated with the more aggressive LC/A phenotype and poor outcomes [46, 58]. Interestingly, SHH and Wnt tumors rarely display abnormalities in chromosome 17 or amplification in *MYC* or *NMYC*.

1.2.3 Current treatments

Current treatments for medulloblastoma are based on risk group-adapted therapies (Table 1-1). Normally, they include surgical tumor resection followed by radiotherapy or chemotherapy, with adapted regimens for high-risk or standard-risk patients. Radiotherapy has shown to be one of the most powerful tools for the treatment of medulloblastoma, but its utility is limited, because of the deleterious side effects caused on the developing brain of young patients that appear at higher doses of irradiation. A subset of medulloblastomas responds to chemotherapy, the most commonly chemotherapeutic agents used being methotrexate, etoposide, cyclophosphamide and platimum derivatives. The combination of chemotherapy and radiotherapy has improved the 5-year overall survival rate to 55% to 76% in high-risk patients and to 70% to 80% in standard-risk patients. Unfortunately, attempts to further reduce disease related mortality have been restricted by the toxicity of the conventional treatments [47].

Variable	**Prognostic Category**			
	Low Risk	Standard Risk	High Risk	
Age	> 3	> 3	> 3	< 3
Pattern of disease	< 1.5cm3 residual disease No Metastasis	< 1.5cm3 residual disease No Metastasis	> 1.5cm3 residual disease Metastasis	
Tumor genomics	Classic or desmoplastic No *MYC* amplification Wnt MB	Classic or desmoplastic No *MYC* amplification Shh or Non Shh/Wnt MB	LC/A *MYC* amplification Wnt, Shh or Non Shh/Wnt MB	
Treatment	Chemotherapy	Reduced dose CSI Chemotherapy	Full dose CSI Chemotherapy	Chemotherapy
Survival rate	> 80 %	80 %	40 to 60 %	20- 40 %

Table 1-2 Patient stratification and risk adapted treatment. Data in this table have been derived from [46] and [59].

1.3 Receptor tyrosine kinase signalling and its implication in cancer

1.3.1 Receptor tyrosine kinases

Receptor Tyrosine Kinases (RTK) play a central role in the regulation of normal cellular processes, such as cell survival and proliferation, cell growth, metabolism, differentiation, cell adhesion and migration [60]. Defects in RTK signalling have been shown to be critical for the development and progression of many types of cancer. The elucidation of the involvement of RTKs in tumorgenesis has led to the design of new drugs that specifically inhibit RTK activity, contributing to the development of the first target-specific cancer therapeutics.

Cells express a variety of RTK, which are divided into 20 subclasses according to their primary structure, ligand affinity and induction of biological response. All RTKs share a common molecular structure with a ligand-binding domain in the extracellular region, a single transmembrane helix, and a cytoplasmic region that contains the protein tyrosine kinase (TK) domain plus additional carboxy (C-) terminal and juxtamembrane regulatory regions [60]. Activation of the receptor occurs upon ligand binding, which induces conformational changes that allow for receptor dimerization. Upon dimerization, autophosphorylation of tyrosine residues within the receptor cytoplasmic domains takes place, generating docking sites for important signal transducers. These molecules contain Src homology 2 (SH2) domains or phosphotyrosine binding (PTB) domains, which either directly bind to phosphotyrosine sites on the receptor or are recruited indirectly by binding to other docking proteins, such as FRS2, IRS1 or Gab-1 [61], which may recruit additional adaptor or signalling proteins. Due to the large number of existing docking, adaptor and signalling proteins, RTKs can clearly recruit multiple protein complexes to the membrane and influence a large number of different signalling molecules. The signalling pathway that is in turn activated depends exclusively on the protein complex which is recruited to the membrane

upon receptor activation. For the content of this thesis, the roles of the fibroblast growth factor receptor (FGFR) and insulin like growth factor (IGF-1R) are of importance and will be discussed below.

FGFR

The mammalian FGF family comprises 18 ligands, which bind to 4 highly conserved transmembrane RTKs (FGFR 1, FGFR2, FGFR3 and FGFR4) [62]. Activated FGFRs provide binding sites for FRS2, a key adaptor protein that is largely specific to FGFRs [63]. Binding of FRS2 allows for the recruitment of the adaptor protein growth factor receptor bound 2 (Grb2), which in turn binds to the guanine nucleotide exchange factor SOS, which activates the RAS GTPases and starts downstream the RAF/MAPK signalling pathway. A separate complex that also involves Grb2 recruits Gab-1 (Grb2 associated binding protein 1) to the membrane and activates the PI3K/AKT signalling pathway. Another main substrate of FGFRs is the phospholipase Cγ (PLCγ), which directly binds to the phospho-tyrosine residues of FGFRs through its SH2 domains. Once PLCγ is recruited to the membrane, it hydrolyzes phosphatidylinositol-4,5-biphosphate (PIP_2) to phosphatidylinositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces the opening of Ca^{2+}-channels and the accumulation of Ca^{2+} in the cytosol. Ca^{2+} and DAG promote the activation of protein kinase C family members (PKC), which partly reinforces the activation of the MAPK pathway by phosphorylating RAF [64]. Several other pathways are also activated by FGFRs, depending on the cellular context, including the p38 MAPK and Jun kinase pathways, signal transducer and activator of transcription (STAT) signalling and ribosomal protein S6 kinase 2 (RSK2) [62].

FGFR regulates fundamental developmental processes during embryogenesis. Deregulation in FGFR signalling results in developmental syndromes, such as skeletal dysplasias. In adult tissue, FGFRs are known to regulate wound repair, angiogenesis, cell proliferation and differentiation, processes that are often deregulated in cancer cells. The importance of FGF signalling was highlighted by a screen for somatic mutations in the human kinome from 210 different human cancers which

identified the components of FGF signalling pathways as the most commonly mutated coding regions [65]. Genetic alterations of *FGFR* have been extensively described in solid tumors and hematologic malignancies, being the most prominent mutations in *FGFR3* and gene amplifications in *FGFR1* and *FGFR2*. Activating mutations in *FGFR3* and *FGFR2* are most frequently found in bladder cancer (in 50% of bladder cancers, which are associated with a non-muscle invasive disease and in 10-15% of the invasive type) (42). Mutations in *FGFR3* are also found at lower rates in cervical cancer, myeloma and prostate cancer. Mutations in *FGFR2* are found in 12% of endometrial cancers and amplification in 10% of gastric cancers, the last one being associated with poor prognosis. Amplifications in *FGFR1* are frequently found in 10-15% of breast cancers and it are often accompanied by increased or altered expression of FGF ligands [66].

IGF-1R

The Insulin-like Growth Factor 1 (IGF-1) Receptor is a transmembrane hetero-tetramer receptor with two extracellular α-subunits and two β-subunits with intrinsic tyrosine kinase activity [67]. The IGF-1R has affinity for IGF-1 and IGF-2, which are polypeptide protein hormones similar to insulin [67]. As other RTKs, ligand mediated activation of IGF-1R results in the autophosphorylation of its tyrosine residues, leading to the recruitment of the adaptor proteins IRS1 and Shc to the receptor β-subunits intracellular domains [68, 69]. This process activates the PI3K/AKT and the RAS/RAF/MEK/ERK pathways resulting in the stimulation of cell growth, cell cycle progression, increased proliferation and inhibition of apoptosis.

The importance of IGF-1R in normal mammalian development is clear from studies in mice lacking functional receptors. IGF-1R null mice are 45% of the size of wild-type mice at the time of birth and die shortly after because of severe organ hypoplasia [70]. Due to its important role in growth and development, it is not surprising that deregulations in IGF-1R signalling lead to malignant transformation. Elevated levels of IGF-1 and IGF-2 have been associated with increased cancer risk (eg. colon, breast, and prostate) [71, 72]. Several studies have demonstrated that an intact

IGF-IR is necessary for cell transformation by many oncogenes [73]. *In vivo* models have demonstrated that IGF-IR expression induces tumor growth and metastasis, whereas decreasing IGF-IR expression leads to decreased tumor growth in the majority of tumors [74]. Indeed, the IGF1R has been frequently found over-expressed in a broad range of adult tumours, including colon, pancreas, prostate, kidney and breast cancer [75-77] and also childhood tumors [78, 79]. To date, there are about 30 drug candidates targeting the IGF-1R, which are being investigated in clinical trials for the treatment for many cancer types [80].

Activation of the PI3K/AKT signalling pathway

The phosphatidylinositol-3-kinase (PI3K) pathway regulates several cellular processes downstream of different RTKs, such as IGF-1R, FGF-R, vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR) in response to growth factor stimulation [81]. PI3Ks represent a family of protein and lipid kinases, which are divided into three classes (I-III) according to their sequence homology and *in vitro* lipid substrate specificity [82]. Among all isoforms, there is much more evidence for a role of class I PI3K in tumorgenesis and, therefore, it became the focus of attention in cancer research over the past decade.

Class I_A PI3Ks are heterodimers consisting of a p110 catalytic and a p85 regulatory subunit. The p110 catalytic subunits p110α, p110β, and p110δ are highly homologous isoforms. The regulatory isoform p85 includes two Src homology 2 (SH2) domains and a p110 binding domain [83]. When activated, class I_A PI3Ks catalyze the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3), which acts as a second messenger supporting the recruitment of the serine/threonine kinase AKT to the plasma membrane by binding to its pleckstrin homology domain [84, 85]. AKT represents a central node in the intracellular signalling networks. Once activated, AKT phosphorylates numerous targets starting a series of signalling cascades involved in diverse cellular functions. AKT inhibits

apoptosis by direct phosphorylation of BAD [86], caspase-9 [87] and FKHR [88]. Additionally, AKT inhibits p53-dependent cell death by phosphorylation of MDM2 [89]. Besides the regulation of the anti-apoptotic pathway, AKT promotes cell cycle progression by the inhibition of glycogen synthase kinase-3β (GSK-3β), leading to the accumulation of cyclin D1 via myc [90]. AKT also promotes cell growth by activation of the rapamycin-sensitive complex mTORC1 [91], which is responsible for the activations of two important regulators of protein translation and ribosomal biogenesis: p70 ribosomal S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) [92, 93].

The PI3K/AKT pathway is antagonized by the lipid phosphatase PTEN, a 3-position phosphatase that converts PIP_3 back to PIP_2 [94, 95], thus controlling the levels of second messenger generated. PTEN is crucial for the regulation of the PI3K/AKT pathway, since there is no other related protein that compensates its loss of function. PTEN dysfunctions have been implicated in a wide range of cancer types, highlighting its importance for the proper regulation of cell growth and proliferation [96].

Activation of the Raf-MEK-ERK pathway

Another key signalling pathway that can be regulated by RTKs is the mitogen-activated protein kinase (MAPK) pathway. All eukaryotic cells use multiple MAPK cascades for signal transduction. Each cascade consists of at least three evolutionary conserved enzymes that are activated sequentially: MAPKK kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAP kinase (MAPK) [97]. MAPK pathways integrate signals from several receptor stimuli and induce a variety of cellular responses, including cell proliferation, differentiation, development, inflammatory responses and apoptosis [98]. Six distinct groups of MAPKs have been characterized in mammals; extracellular signal-regulated kinase ERK1/2, ERK3/4, ERK5, ERK7/8, Jun N-terminal kinase (JNK) 1/2/3 and the p38 isoforms (ERK6) [99]. The best characterized MAPK signalling pathway represents the Raf-MEK-ERK pathway, which is deregulated in approximately one third of all human cancers [99]. This pathway is

activated by the GTPase Ras, a critical link between RTKs and ERK. Upon ligand binding, Grb2 and the guanine nucleotide exchange factor SOS are recruited to the plasma membrane. SOS promotes the exchange of GDP to GTP and the activation of Ras, which can directly phosphorylate Raf (Raf-1, B-Raf and A-Raf) to initiate the cascade. Activated Raf activates MEK1 and MEK2, which subsequently activate ERK1 and ERK2. The ultimate targets of ERK are a variety of subtrates including membrane proteins, cytoskeletal proteins and transcription factors [100]. The type of process that is regulated as a consequence of the RAF/MEK/ERK axis depends exclusively on the targets that ERK binds to. These properties might be linked to temporal differences in the strength and localization of ERK within the cell [100].

Most of the alterations leading to constitutive activation of ERK signalling occur at early steps of the pathway, either from aberrant signalling from RTK or from activating mutations in Ras and B-Raf. Ras and B-Raf mutations are in most of the tumors mutually exclusive, and mainly found in Ras, which was one of the first oncogenes identified. Activating Ras mutations are reported in pancreas- (90%), thyroid- (55-60%) non-small cell lung- (35%) and liver (30%) cancer, among others [101]. The high incidence of activating mutations in the Ras–Raf axis points for its role as regulatory hotspot [99].

Protein kinase C

Protein kinase C (PKC) is a family of serine/threonine kinases consisting of nine isozymes: classical (PKCα, PKCβI, PKCβII, and PKCγ), novel (PKCδ, PKCε, PKCη, and PKCθ), and atypical (PKCζ and PKCι) [102]. They are divided into three subfamilies based on their second messenger requirements: classical PKC requires calcium, phorbol esters or diacylglycerol (DAG) for its activation, novel PKCs are activated by phorbol esters or DAG and atypical PKCs are unresponsive to calcium, phorbol esters or DAG. Thus, classical and novel PKCs are activated through the same signal transduction pathway as PLC. The structure of all PKCs consists of a regulatory domain and a highly conserved catalytic

domain [103]. The second messenger requirement differences in the isoforms are a result of the regulatory region, which are similar within the classes, but differ among them.

PKCs were originally thought to be pro-mitogenic kinases, but later it was shown that this effect is isoform- and cell-type dependent [102]. Upon PKC activation, which normally occurs at the plasma membrane, the different PKC isoforms redistribute to the nuclear membrane or to different organelles. The differential redistribution is the key for dictating substrate specificity and thus functional selectivity [104].

The role of PKC in tumorigenesis was first described because of the observation that phorbol esters promoted tumor formation on a mouse-skin model. For many decades, PKCs have been linked to cancer, but the role of the specific isoforms is still very controversial [104].

PKCs have shown to have opposing roles in the regulation proliferation, survival and apoptosis, which is particularly interesting for the widely expressed PKCε and PKCδ isoforms. PKCε promotes cell survival in many cell types through increased activation of the Akt pathway and up-regulation of pro-survival factors. Over-expression of PKCε was also associated with resistance to chemotherapy and invasiveness [105]. PKCδ has been shown to have a dual role as both, positive or negative growth regulator. It was shown to induce cell cycle arrest and promote apoptotic signals in various cell types [106]. In another cellular context, PKCδ was shown to positively regulate proliferation, for example by stimulating the ERK pathway in breast cancer cells [107]. Also, PKCδ has been shown to be required for the proliferative effect of insulin-like growth factor 1 (IGF1) [108].

There is a limited number of mutations identified in PKCs in human's cancer [88]. However, altered levels of PKC isoforms have been shown to correlate with several cancer-associated phenotypes, such as cell survival and proliferation or cell migration and invasion.

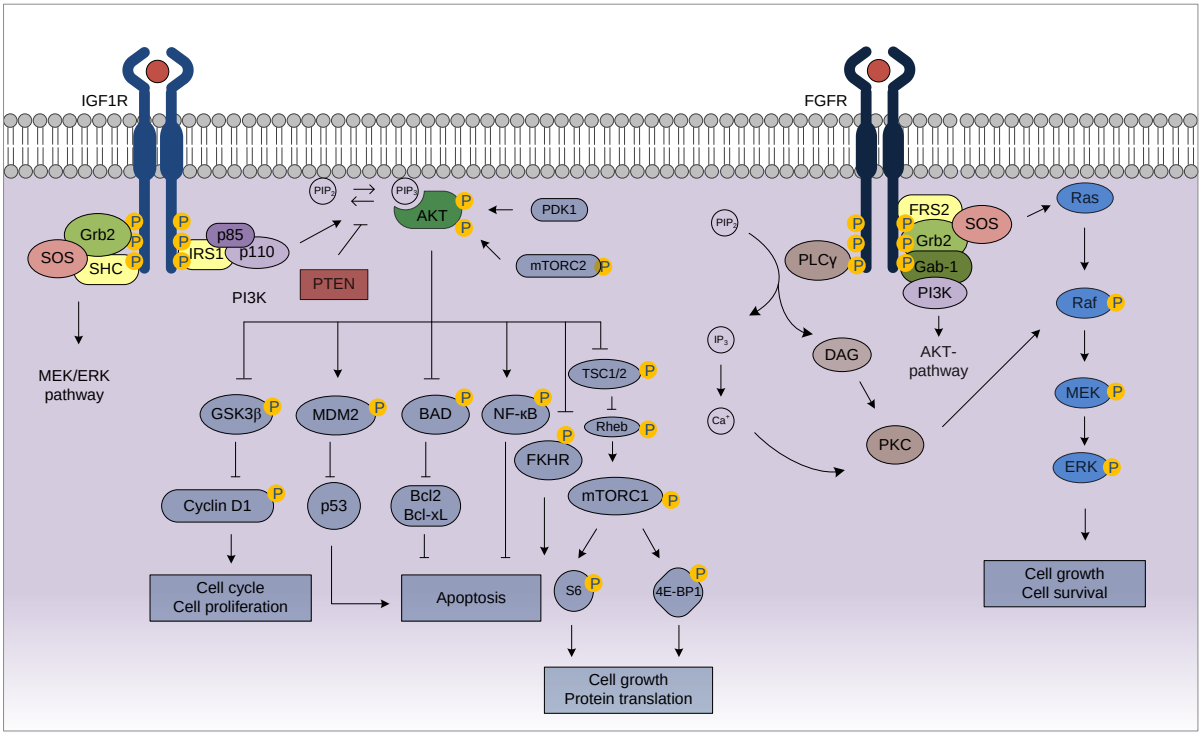

Figure 1-2. Schematic representation of receptor tyrosine signalling networks. FGFR and IGF-1R are activated by autophosphorylation in response to growth factor binding and recruit different docking or adaptor proteins to initiate the PI3K/AKT and the RAF/MEK/ERK signalling cascades, which are involved in the regulation of cell growth, cell proliferation and apoptosis.

1.3.2 Genetic alterations in the RTK signalling networks in cancer

Due to the central role of the RTK network in the control of cell proliferation, cell survival and cell death, it is not surprising that alterations in its components, which lead to increased pathway activation, result in an uncontrolled cell proliferation and malignant transformation. Extensive studies performed during the past years have identified numerous mutations or gene amplifications that target RTKs or components of downstream signalling pathways, such as the PI3K/AKT and MAPK pathway, in a number of human cancers [81, 109, 110]. The focus of this section is on the main genetic alterations in RTKs and its

downstream signalling components in neuroblastoma and medulloblastoma.

Neuroblastoma

Receptor tyrosine kinases

Among RTKs, ALK is probably the most frequently altered in neuroblastoma, accounting for 5 to 15% of neuroblastomas. Gain-of function mutations in *ALK* have been identified in conserved positions of the tyrosine kinase domain [13]. From the 12 residues identified, the most common mutational hotspots are R1275Q and F1174L. The R1275Q mutation is found in familial and sporadic neuroblastomas, whereas F1174L is restricted to sporadic tumors and is associated with amplification of the *MYCN* oncogene [111]. Gene amplifications in *ALK* have been also reported, but at lower frequencies than point mutations (2% of all neuroblastomas) [111]. Interestingly, it was shown that *ALK* amplifications occur almost exclusively with MYCN amplification [112]. The Trk receptor family has been shown to be differentially expressed in neuroblastomas and is frequently used as a prognostic marker in neuroblastoma. Overexpression of TrkA is characteristic for tumors with favorable biology, while TrkB expression correlates with *MYCN* amplified tumors and aggressive disease [113]. Several polypeptide growth factors have been shown to promote neuroblastoma proliferation and chemoresistance. Early studies identified IGF-I and IGF-II signalling loops to play a role in neuroblastoma cell survival [114-116]. IGF-IR expression has been shown to correlate with neuroblastoma tumorigenicity [117]. FGFR expression was only investigated in NB cell lines, in which FGFR1 expression and activation by FGF-2 was associated with *MYCN* expression [118].

Raf/Mek/Erk

Although the Ras-Raf axis represents a hotspot for activating mutations in a high percentage of human cancers, they occur very infrequently in neuroblastoma [119-121]. Among other Ras effector genes that may also contribute to tumorigenesis, the RAS-association domain family 1 isoform

A (RASSF1A) has been identified to be epigenetically silenced in a significant proportion of high-risk neuroblastomas [122]. Although genetic alterations in the ERK pathway are rare, its involvement in mediating aberrant RTK signals has demonstrated to have a crucial role in neuroblastoma progression [123].

PI3K

Also mutations in the *PIK3CA* gene have been extensively detected in a number of human cancers, but, in neuroblastoma, this event seems to be rare (2-3% of the cases) [124, 125]. In two independent studies, *PIK3CA* mutations were found in the catalytically protein domain indicating a possible increase in the p110α activity [124, 125]. However, expression of p110α was detected on a protein level in 92% of paraffin-embedded tissue sections [124]. As in the case of *PIK3CA*, genetic alterations in *PIK3CD* have been shown to be rare [126]. The expression of *PIK3CD* was later shown to correlate with patient age. Elevated mRNA expression of *PIK3CD* was found in patients younger than one year [127]. Currently, no genetic alterations have been reported in *PIK3CB* encoding the class I_A PI3K isoform p110β.

PTEN

In neuroblastoma, alterations involving the *PTEN* gene has been rarely reported [124, 128, 129]. Reduced expression of PTEN seems to be an infrequent event in neuroblastoma. In addition, no associations with aggressive tumor biology could be demonstrated [129].

Medulloblastoma

Receptor tyrosine kinases

Several studies have shown that growth factor signalling plays a key role in medulloblastoma biology. In fact, human medulloblastoma cells express a variety of growth factor receptors, amongst them ErbB-2, ErbB-4, IGF-IR, platelet-derived growth factor (PDGFR), epidermal growth factor receptor (EGFR) and neurothrophin-3 receptor Trk were associated with medulloblastoma carcinogenesis [130-132]. Over-expression of ErbB-2,

ErbB-4 was reported in medulloblastoma tumor samples, and it was associated with reduced patient survival [130]. The expression of TrkC was as well correlated with patient survival, although in this study, increased expression of TrkC represented a marker of good prognosis. This might be explained because of its role in the regulation of apoptosis [131]. Further studies showed that IGF-1R is overexpressed and activated in medulloblastoma. Interestingly the expression of IGF-1R had a strong inverse correlation with TrkC expression [132].

Raf/MEK/ERK

As in the case of neuroblastoma, there are only few studies that address a mutational analysis of the ERK pathway in medulloblastoma. While an early study identified a mutation in NRAS in a single MB cell line [133], a bigger study comprising 28 primary tumors detected no oncogenic mutations affecting NRAS, KRAS, HRAS, BRAF in any case [134], which suggested that alternative mechanisms are responsible for RAS/MAPK pathway activation in this disease. However, as in neuroblastoma, promoter hypermethylation was identified in the RASSF1A gene [135], suggesting a role in the development of embryonal tumors.

PI3K isoforms

Although the PI3K/AKT pathway has been extensively studied in many cancer types, relatively few data are available in medulloblastoma. A large-scale mutational analysis identified mutations in the *PIK3CA* gene in only 5% of medulloblastomas [136] and *PIK3CA* gene amplification was not detected [136, 137]. The different class I_A PI3K isoforms were shown to have differentially expression patterns in medulloblastoma patient samples. Significant over-expression of *PIK3CA* gene in medulloblastoma tumor samples was found compared with normal cerebellum [137]. Additionally, protein over-expression of the PI3K catalytic isoform p110α was observed in human medulloblastoma samples (74%). On the contrary, significant increases in the expression levels of the remaining class I_A PI3K isoforms (p110β, p110δ and p85) were not detected [137].

The activation of the PI3K/AKT signalling pathway has emerged as an important element of medulloblastoma cell proliferation [137-139] and a key role in this pathway has been attributed to class I_A PI3K p110α. The importance of p110α was proven, as its inhibition led to a decrease in cell proliferation and augmented the effects of chemotherapy in a cell line model [137].

PTEN

Different studies reported that *PTEN* expression is reduced in medulloblastoma [138, 140]. In primary medulloblastoma samples, *PTEN* mRNA and protein expression were found significantly lower when compared to normal cerebellar tissue, independently of medulloblastoma histological subtype. In 50% of the tumor samples, reduction of *PTEN* expression was found to be associated with PTEN promoter hypermethylation [138]. *PTEN* homozygous loss at chromosome 10q was detected in 32% of medulloblastomas [140]. The fact that only a subset of samples lack of *PTEN* expression indicates that loss of *PTEN* does not seem to be a common mechanism in medulloblastoma development. In agreement with this, a different study found that the PTEN protein was expressed at higher levels in medulloblastoma samples and cell lines as compared to control samples [137].

1.4 Targeting the human kinome in cancer

1.4.1 Rationale for targeting kinases in cancer

Tumorigenesis is a multistep process that involves dynamic changes in the genome of normal human cells, driving their progressive transformation into highly malignant derivatives [141]. Cancer cells can accumulate an immense variety of defects in the regulatory circuits that govern normal cell proliferation and homeostasis, that ultimately lead to malignant growth. Understanding these dynamic variations is crucial to develop novel targeted therapies to inhibit malignant tumor growth.

Most of cellular processes are controlled by the reversible phosphorylation of distinct signalling proteins by protein kinases or phosphatases [142]. Up to 30% of all human proteins may be modified by phosphorylation [143]. The enzymatic activity of kinases is therefore under tight control, non proliferating cells having very low levels of phosphorylated proteins [142]. At least 518 distinct kinase-encoding genes in the human genome have been identified, from which 164 map to amplicons in cancer [144]. Given the large number of existing kinases and their different levels of regulation, it is not surprising that they are frequently deregulated in cancer.

Alterations in kinase signalling can occur at many levels (Figure 1-3) [142]. Increased kinase activity can result from a gain-of-function mutation that disrupts auto regulation of the kinase and renders it constitutively active, independent of upstream signalling. For example, mutations in the tyrosine kinase domain of epidermal growth factor receptor (EGFR), that alter receptor signalling, have been identified in a subset of non–small-cell lung cancers [145]. Not only activating mutations, but also over-expression of receptor tyrosine kinases or downstream protein kinases can lead to aberrant signalling. Such is the case for the receptor tyrosine kinase ERBB2 (HER-2/neu) in breast cancer [146]. Lastly, increased kinase activity can result from loss-of-function

alterations in factors that antagonize kinase activity, such as phosphatases or inhibitory proteins. The most renowned example is probably the tumor suppressor *PTEN*. Loss-of-function mutations in *PTEN* are frequently found in many human cancers [147], which underlines the importance of a proper control mechanism for the maintenance of cell homeostasis.

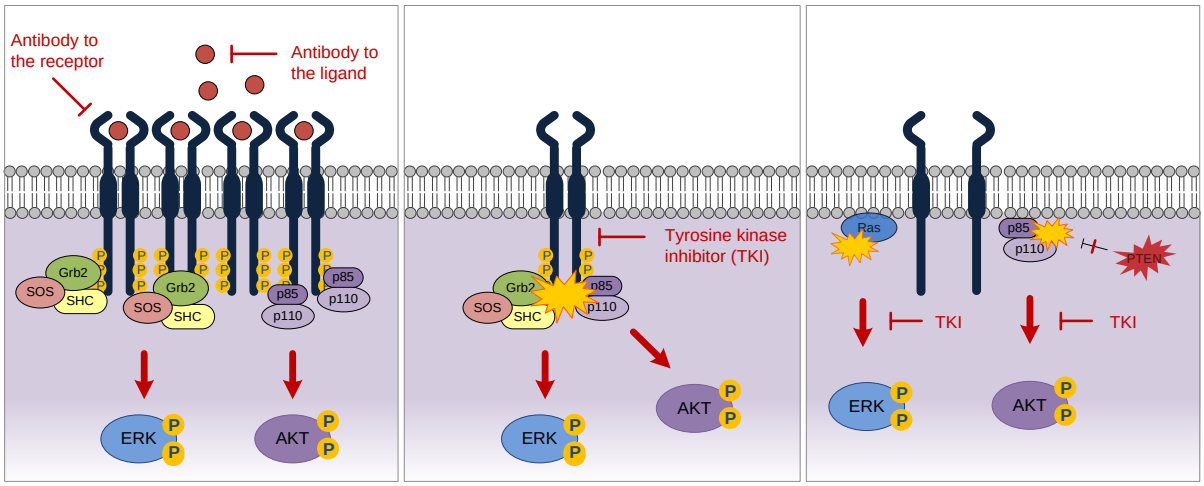

Figure 1-3. Alterations in RTK signalling. Over-expression of receptor tyrosine kinases or its ligands (right), activating mutations on the receptor (middle) or in downstream signalling factors (left) lead to aberrant RTK signalling. The possibilities to inhibit the signalling pathway are shown.

Knowing the possible molecular mechanisms of grown deregulation in cancer cells and which key factors are involved in them, opened the possibility for researchers to design new treatment strategies based on targeting specific proteins which are responsible for an oncogenic phenotype. Tyrosine kinases emerged very soon as druggable targets, since their activity can be inhibited pharmacologically in several ways [142]. Small molecule inhibitors for example can bind at the catalytic site of the kinase, preventing the binding of ATP or substrates. Other kinase inhibitors function by blocking the dimerization of protein subunits to inhibit their activation. In addition to small molecule inhibitors, it is also possible to block signal transduction with antibodies against receptors or their ligands. They function through neutralization of ligand, inhibition of

ligand binding and/or receptor internalization. The large number of kinases known to be altered in cancer and all the possibilities to inhibit their function led to the development of an arsenal of pharmacological inhibitors and antibodies, which have been and are being tested in both preclinical and clinical settings.

1.4.2 Targeting RTK signalling in neuroblastoma and medulloblastoma

ALK

Due to the high incidence of mutations in the *ALK* gene that have been identified in neuroblastoma, ALK has emerged as a novel promising therapeutic target [111]. Indeed, crizotinib an ALK inhibitor has recently entered the phase I clinical trial for the treatment of neuroblastoma and other solid tumors (clinical trial ID: NCT00939770, [46]). Preclinical studies have shown that neuroblastoma cell lines with ALK amplification or the R1275Q mutation are sensitive to crizotinib, but not those cell lines expressing the F1174L mutation [111]. On the contrary, neuroblastoma cell lines bearing the F1174L mutation were sensitive to another inhibitor named TAE684 [148]. Due to the different responses to ALK inhibitors and also the possible emergence of drug resistance, there is currently a big interest in searching for new ALK inhibitors [111], some of them are being identified form high-throughput screening against multiple RTK targets [149].

IGF-1R

The observation that NB cell lines secrete and respond to several IGFs to promote cell proliferation, has led to the design of anti-tumor strategies based on inhibiting the activity of IGF-1R. Several pharmacological inhibitors and neutralizing antibodies that inhibit IGF-1R signalling have been tested *in vitro* and *in vivo* and demonstrated anti-proliferative effects: the monoclonal antibodies SCH717454 [150, 151] and EM164 [152], AMG479 [153], and the tyrosine kinase inhibitors NVP-AEW541 [154, 155], CP-751,871 [156] and BMS-554417 [157]. Due to the promising

results obtained in preclinical models, the anti-IGF-1R antibodies SCH717454 and Cixutumumab are currently being tested in Phase I and Phase II clinical trials (NCT00831844, NCT00960063 [46]).

The therapeutic potential of inhibiting IGF1R signalling in medulloblastoma has been also studied [158-160]. For example, inhibitors NVP-ADW742 and NVP-AEW541 attenuated growth in medulloblastoma cell lines [160]. Interestingly, MB cell lines which showed resistance to NVP-AEW541, were associated with phosphorylation of GSK3 and could become much more sensitive following SNP-mediated GSK3 dephosphorylation [160].

EGFR

Given that medulloblastoma cells express ERBB2, ERBB4 and EGFR, the therapeutic potential of the inhibitors erlotinib and genitinib was assessed *in vitro* and *in vivo* [161-163]. Because of the anti-tumors effects observed in preclinical models, erlotinib entered Phase I and II clinical trials [46, 161, 164]. Although erlotinib was well tolerated by children, only few showed a modest response to the treatment [164]. Recently, a dual inhibitor of HER1/2 and VEGFR1/2 named AEE788 showed anti-tumor activity in medulloblastoma xenografts [162]. Importantly, the subset of HER2-overexpressing medulloblastomas was shown to benefit most likely from AEE788 treatment, highlighting the importance of the identification of tumour markers to predict drug response.

PI3K/AKT/mTOR

Because of the fact that tumors express more than one RTK to activate downstream signalling pathways, targeting individual receptors may not always yield optimal results in inhibiting cell proliferation. A possible approach to overcome resistance to RTK inhibitors is targeting protein kinases that lie downstream of the several different growth factor receptors and are required for transmitting proliferative signals from the receptors. PI3K represents an attractive target for pharmacological inhibition, therefore many inhibitors have been developed and widely studied in

many cancer types [109]. One of the most prominent PI3K-inhibitors is PI-103, which has dual specificity against the isoform p110α and mTOR. PI-103 inhibited neuroblastoma growth in vitro and in vivo [165, 166]. In medulloblastoma, inhibition of the PI3K isoform p110α with the isoform specific inhibitors PIK75 and YM-024 were reported to have anti-proliferative effects in medulloblastoma cell lines [137]. Inhibition of PDK1 with OSU03012 showed also cell growth inhibition in NB and MB *in vitro* and *in vivo* models [167]. Finally, the AKT inhibitor perifosine has as well shown anti-tumor effects, although so far only *in vitro* in MB and NB cell lines [168, 169].

1.4.3 Resistance to kinase inhibitors

Although many TKIs showed to be able to inhibit cell survival and proliferation in preclinical settings, their use for patient treatment showed to be challenging [170]. One of the reasons may be that *in vitro* and *in vivo* models rarely represent the vast genetic heterogeneity of tumors occurring in patients. In addition, tumor cells can develop a number of mechanisms to circumvent drug activity. The identification of tumor subgroups that share genetic features will certainly help to choose appropriate treatments for each patient subgroup. Important lessons could be learned from studies performed with the EGFR inhibitors erlotinib and gefitinib for the treatment of glioblastoma (reviewed in the appendix). *EGFR* mutation and amplification represent the most prominent genetic alterations in malignant brain tumors [171]. Responsiveness of glioblastoma patients to EGFR inhibition correlated with expression of EGFRvIII, a constitutively active mutant of EGFR [172]. This study is of great importance, because it exemplifies how activating mutations might render cancer cells oncogenically addicted to a specific pathway and thus, sensitive to small molecule inhibitors targeting the specific oncogenic factor. Interestingly, the same study identified a subgroup of glioblastomas that did not respond to EGFR inhibition and their common genetic feature was loss of *PTEN* expression [172]. Another study in glioblastoma showed that response to erlotinib was also associated EGFR over-expression, but only in the

subgroup of glioblastomas with basal AKT phosphorylation [173]. None of the patients with tumors expressing high levels of activated AKT responded to erlotinib. Together, these studies exemplify some of the multiple mechanisms that cancer cells may use to overcome target inhibition.

To date, numerous mechanisms of therapy resistance have been described in the literature. The majority of cancers are genetically complex: they normally use more than one signalling pathway to drive malignant growth. Therefore, their survival not solely depends on the activation of a single molecule or a receptor and it is unlikely that single agent treatment will induce complete inhibition of cell growth [174]. It has been observed that multi-targeting therapies show more potent effects in this context, and may represent a more promising option for patient treatment [174].

1.4.4 Design of drug combination approaches

Despite all the advances achieved in cancer biology, conventional chemotherapy targeting the DNA of proliferating cells is still most efficacious for cancer treatment. Although often limited by toxicity, chemotherapy has shown good responses for many cancer types [175]. For most of advanced tumors single drug treatment still represents a problem. Even though advanced tumors may initially respond, their genetic instability allows for the emergence of secondary mutations and alterations in their genome that leads to a more malignant phenotype with acquired resistance to the initial treatment [176]. To address this problem, several drug combinations have been tested and shown to be more effective in curing cancers. Therefore, many combinatorial regimens have been integrated in the clinic for the half past century, and in some cases they have yielded complete disease remission [176]. Since most of the conventional chemotherapies have, in principle, the same mode of action, it is likely that when cancer cells develop a mechanism of resistance to any agent, they will simultaneously acquire multi-resistance to other agents without being treated with them at first place [177]. The

identification of kinases that modulate the response to drug treatment provides a rational to design new therapeutic approaches, based on the combination of conventional chemotherapies with small molecule inhibitors. Indeed, some studies have prescuted some valuable combinations that are currently being tested in clinical trials [46]. Although this strategy is very promising, it remains challenging to identify the optimal combination for a certain type of tumor. The search for the adequate combination might be very time consuming and expensive. The use of genome-wide high-throughput screens has greatly facilitated the identification of molecular signatures that modulate the response of cancer cells to anticancer drugs. RNA interference (RNAi) screens have shown to have great potential in this context and are described in more detail in the next section.

1.4.5 RNA Interference to enhance cancer drug target discovery

RNA interference (RNAi) is a natural gene silencing mechanism that all organisms use as a post-transcriptional regulation of their gene expression. RNAi is considered to be an evolutionary ancient mechanism for protecting organisms from viruses [178]. Since many viruses use RNA, instead of DNA, as their genetic material, eukaryotic cells have evolved to recognize this double stranded RNA and to induce its degradation as an antiviral response. Since its discovery, the molecular mechanisms of RNAi have been extensively studied [179]. The RNAi response starts when an enzyme called dicer recognizes dsRNA and degrades it into small segments around 20 nucleotide pairs in length. A protein complex called RISC (RNA induced silencing complex) binds to the small RNA segments and uses one of its strands as a template to seek out mRNA transcripts with the complementary sequence and prevent its expression. The fact that RNAi works in a sequence-specific manner to degrade the target mRNA has enormously facilitated the design and development of synthetic small interfering RNA molecules (siRNA) to specifically ablate the function of any gene in the genome [180].

RNA interference is now commonly used to study the effects of blocking the expression of a gene of interest [181]. With the completion of the human genome sequencing and DNA microarray technologies, huge databases have been generated that led to the identification of several groups of genes which are deregulated in various diseases, but no information about the function of these genes was provided. The ability of RNA interference to induce gene down-regulation in a relative easy manner, has opened up the possibility to systematically target every gene of interest and to simultaneously probe gene function on a large scale [181]. This led to the development of several RNAi libraries, which are composed of collections of small interfering RNAs that can be genome-wide or can be designed on a smaller scale to target a subgroup of genes (eg kinome-wide). RNAi libraries are nowadays produced by several commercial and academic laboratories. They are reliable and robust, contain several unique validated sequences per gene and are usually pooled in a 96- or 384- well plate format to perform high throughput screens. Commonly used readouts for arrayed libraries are luminescent cell viability or apoptosis assays, but also methods involving more sophisticated microscopic image analysis have been reported [182]. Several loss-of-function RNAi screens have been conducted in human cancer models in the past few years, and have reported novel key regulators of various cancer-related phenotypes [183-186].

RNAi are well suited for the identification of target genes that promote cell growth and are required for survival, as their specific silencing will potentially result in a selective decrease in survival of the cells, which were transfected with the respective siRNA. RNAi screens of this type identified survivin as a target in a colon carcinoma cell line harboring the activated KRAS oncogene [187], or PLK1 as a potential target in a medulloblastoma cell line [188]. RNAi screens can be well performed in combination with standard chemotherapeutic drugs. The so called RNAi chemosensitization screens have the ability to identify targets which are responsible for mediating drug resistance and help the design of drug-combination strategies. Several recent studies have reported on RNAi

chemosensitization screens [184, 189, 190]. A genome-wide RNAi screen identified genes that modify the response to paclitaxel in non-small-cell lung cancer. The hit candidate genes included proteasome subunits, which when being inhibited by bortezomib had a synergistic effect with paclitaxel in patients [182]. This study highlights the use of RNAi screens to identify effective combination therapy targets. Similarly, other RNAi screens identified FER (fps/fes related tyrosine kinase) to sensitize HeLa cells to cisplatin, paclitaxel and etoposide [184], or MAPKAP1 to sensitize pancreatic cancer cells to gemcitabine [189].

There is a growing number of RNAi chemosensitization screens reported, and a number of novel combination approaches proposed, which proves the potential of RNAi screening technologies, but also points out the importance for proper target validation in order to find druggable targets that can be used in a clinical setting.

1.5 Aims of the thesis

Receptor tyrosine kinases and their downstream signalling cascades have been shown to play a crucial role in human cancers. Promising new cancer therapies are emerging from targeting signalling molecules with selective pharmacological inhibitors, in order to attenuate the survival or proliferative message in the cell. Therefore, it is important to identify which signalling pathways, and especially which components on those pathways are responsible for rendering cancer cells the oncogenic phenotype. The overall goal of the present thesis was to contribute to the knowledge of the molecular mechanism underlying cell survival and chemoresistance in two embryonal tumors: neuroblastoma and medulloblastoma.

In the main project, a large-scale approach was designed that aimed for the screening of the human kinome in neuroblastoma to uncover kinases which are critical for promoting cell survival. Knowing that drug resistance is a major cause of treatment failure in high risk neuroblastoma patients, we performed a chemo-sensitizing kinome-wide RNAi screen to systematically identify new determinants of resistance to cisplatin, a commonly used therapeutic agent for the treatment of neuroblastoma. The screen was performed in two neuroblastoma cell lines with different *MYCN* status, in order to assess the differences in the sensitivity to cisplatin and to find target genes for each subgroup. After identification and validation of the candidate kinases, specific small molecule inhibitors against the candidate kinases were used to investigate whether the RNAi-mediated effects observed on cell survival and response to cisplatin could be reproduced by means of pharmacological targeting of the kinases. An identical loss-of-function RNAi screen has previously been performed by our group for medulloblastoma. This study validated a novel role for PI3K-p110γ in medulloblastoma chemoresistance and suggested a rational to combine pharmacological inhibitors against p110γ with chemotherapeutic agents.

The second project is based on observations of previous work from our group. In previous studies, distinct roles of the PI3K isoforms in neuroblastoma and medulloblastoma have been investigated. In neuroblastoma, a novel function for the isoform p110δ in cell growth and survival was demonstrated. Down-regulation of either p110α or p110δ led to impaired cell growth, although the sensitivity of the isoform down-regulation was cell type dependent. In medulloblastoma, p110α was shown to play the main role among all PI3K isoforms. Over-expression of isoform p110α was detected in a panel of medulloblastoma cell lines and tumour samples. Inhibition of p110α resulted in a decrease in cell growth and cell proliferation. Interestingly, a stronger decrease was observed by simultaneous down-regulation of p110α and p110δ. A better understanding of the mechanisms of action of p110α and the downstream effectors that contribute to oncogenic p110α signalling may lead to the identification of new therapeutic targets. Aiming at discovering new PI3K target genes, we performed a cDNA microarray analysis to compare the changes in the gene expression profiles of medulloblastoma cells caused by RNAi-mediated down-regulation of p110α, p110δ and their combination. With the use of bioinformatic tools, we analyzed transcriptional networks of genes that may be affected by the down-regulation of the PI3K isoforms. Having identified these networks, we sought to analyze their contribution to medulloblastoma survival and proliferation. The expression of the validated target genes was assessed and, finally, the anti-proliferative effects of its inhibition in medulloblastoma cells were analyzed. With such studies we explored the pharmacological potential of the newly identified genes.

In a third project, the goal was to further analyze the therapeutic potential of targeting the axis of the insulin-like growth factor-1 receptor (IGF-1R) and PI3K signalling in medulloblastoma and neuroblastoma. For this purpose, we used R1507, an IGF-1R specific humanized monoclonal antibody, and PIK75, a specific PI3K p110α inhibitor. Following inhibition of IGF-1R or p110α in NB and MB cell lines, the effects on cell proliferation and apoptosis were analyzed. In addition, we analyzed the

sensitization potential of PIK75 or R1507 to commonly used chemotherapeutic agents.

Taken together, the objective of this thesis was to investigate the mechanisms of action of already known oncogenes, such as PI3K-p110α, and to further test the therapeutic potential of targeting them with pharmacological inhibitors. A second goal was the discovery of new target genes that are essential for promoting oncogenesis in medulloblastoma and neuroblastoma by using high throughput approaches.

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CHAPTER 2

RNA INTERFERENCE SCREENING IDENTIFIES A NOVEL ROLE FOR AUTOCRINE FIBROBLAST GROWTH FACTOR SIGNALLING IN NEUROBLASTOMA CHEMORESISTANCE

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This manuscript is in revision at *Oncogene*.

In this manuscript I performed all the experiments, except those in figure 8g.

2.1 Abstract

Chemotherapeutic drug resistance is one of the major causes for treatment failure in high risk neuroblastoma (NB), the most common extra cranial solid tumor in children. Poor prognosis is typically associated with *MYCN* amplification. Here, we utilized a loss of function kinome-wide RNA interference screen to identify genes that cause cisplatin sensitization. We identified FGFR2 as an important determinant of cisplatin resistance. Pharmacological inhibition of FGFR2 confirmed the importance of this kinase in NB chemoresistance. Silencing of FGFR2 sensitized neuroblastoma cells to cisplatin induced apoptosis, which was regulated by the downregulation of the anti-apoptotic protein BCL2. Mechanistically, FGFR2 was shown to activate PKC-δ to induce BCL2 expression. FGFR2, as well as the ligand FGF-2, were consistently expressed in primary NB and NB cell lines, indicating the presence of an autocrine loop. Expression analysis revealed that *FGFR2* expression correlates with *MYCN* amplification and advanced stage disease, demonstrating the clinical relevance of FGFR2 in neuroblastoma. These findings suggest a novel role for FGFR2 in chemoresistance and provide a rational to combine pharmacological inhibitors against FGFR2 with chemotherapeutic agents for the treatment of neuroblastoma.

2.2 Introduction

The development of drug resistance is a major cause of treatment failure in many cancer types. Tumors can exhibit intrinsic drug resistance before initial therapy or acquire drug resistance through chronic drug exposure [1]. The multiple mechanisms leading to chemoresistance are subject of intensive study, but are not yet fully understood. Cisplatin is a platinum-based DNA-damaging cytotoxic agent [2]. It is one of the most potent antitumor agents and is widely used for the treatment of solid tumors including testicular, ovarian, colorectal, lung and head and neck cancers [3, 4]. Cisplatin induces apoptotic cell death, which results from DNA damage-mediated responses. However, several mechanisms to circumvent cisplatin mediated cell-death have been described, which limit its clinical efficacy as an anticancer drug. These mechanisms can occur before cisplatin binds to the DNA like reduced drug uptake to the cell, drug efflux or drug inactivation through GSH or metallothioneins [2, 5-7]. Resistance to cisplatin can also occur by mechanisms that affect the recognition of the DNA damage signal, like inactivation of TP53 [8], attenuation of the apoptotic response mediated by caspase inactivation [9] or overexpression of antiapoptotic BCL2 family members [10, 11]. Alterations in survival pathways are as well associated with drug resistance, for instance overexpression of HER-2/neu and over activation of PI3K/Akt pathway. Although not directly induced by cisplatin, these alterations compensate for cisplatin-induced cell death signals and contribute to a chemoresistant phenotype [12, 13].

Neuroblastoma is an embryonal tumor that originates from developing neural crest tissues [14]. It is the most common extracanial solid tumour and is responsible for 15% of all cancer-related deaths in childhood. Neuroblastomas show very diverse clinical behavior, ranging from a widely disseminated and highly aggressive disease to tumors that differentiate or spontaneously regress with little or no therapy. Amplification of the MYCN oncogene is found in

approximately 20% of neuroblastoma and correlates with poor disease outcome [15]. Current treatments for high-risk neuroblastoma patients include intense multimodality chemotherapies, including cisplatin, doxorubicin and vincristine [16, 17]. Although most high-risk neuroblastomas initially respond to therapy, 50 to 60% have a relapse with the appearance of more drug-resistant tumors. The identification of molecular mechanisms that lead to drug resistance may open a new window for the design of potential combination therapies to treat this patient group.

Kinases control the phosphorylation of proteins and lipids, which are important to mediate cell survival signals in the cells. They are often deregulated in cancer cells, thus targeting kinases offers a rational strategy to inhibit cell survival and to overcome drug resistance. RNA interference (RNAi) libraries allow high through-put screens, which are based on systematically silencing gene expression in order to identify which genes are essential for a specific function in the cell [18]. Such screens have already proven to be a highly effective research tool for the identification of key determinants of drug sensitivity [19-21]. Here, we performed a kinome-wide RNAi screen to identify kinases that, when being downregulated, sensitize neuroblastoma cells to cisplatin induced cell death. We report that RNAi targeting of *FGFR2* caused the strongest impact in cisplatin sensitization. Moreover, our data show that FGFR2 induces BCL2 expression through activation of PKC-δ, which suggests that overexpression of BCL2 contributes to cisplatin resistance in neuroblastoma and thus, provides evidence of the importance of FGFR2 signalling in neuroblastoma chemoresistance.

2.3 Material and methods

Cell culture and reagents

Neuroblastoma cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1% Penicillin/Streptomycin (Sigma, Buchs Switzerland). All human neuroblastoma cell lines were kindly provided by Dr Brodeur, Children's Hospital of Philadelphia, except LAN-1R which was provided by Dr. N. Gross (CHUV, Lausanne). The compounds SU-5402, Rottlerin, PKCβ1 inhibitor, PKCζ inhibitor, LY-29402, PD-98059, FGF-2 were purchased from Calbiochem (Darmstadt, Germany), Enzastaurin was purchased from Selleck Chemicals (Houston, TX, USA) and ABT-737 from Chemietek (Indianapolis, IN, USA).

HTS Method

Neuroblastoma cell lines (10^4 cells per well for LAN-1 and 7.5 x 10^3 cells for SH-SY5Y) were plated in 96-well plates and transfected 24h later with siRNA (Silencer Kinase siRNA Library Ambion, Applied Biosystems, Foster City, CA, USA) at a final concentration of 30 nM, using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The siRNA library used contained 2157 unique small interfering RNAs (siRNAs) targeting each of the 719 human kinase genes with 3 individual siRNAs per gene. Additional controls were added to each plate of the library. Negative controls: siRNA for GAPDH (Ambion), siCONTROL non-targeting siRNA Pool (Dharmacon, Waltham, MA, USA), ONTarget Plus non targeting siRNA (Dharmacon), and positive controls: siRNA for PLK1, EG5 and siCONTROL TOX (Dharmacon). Twenty-four hours following transfection, one replica plate was treated with cisplatin (2.5 µM for LAN-1 and 0.75 µM for SH-SY5Y, Bristol-Myers Squibb, New York, NY, USA) and one replica plate with 0.01% NaCl vehicle in media. Cell viability was assessed after further 48h using Cell Titer

96 Aqueous One Solution Cell proliferation Assay (Promega, Madison, WI, USA) as per manufacturer's instructions. The screen was performed in triplicate. To determine the sensitivity to cisplatin, log2 ratios for each siRNA were computed from the mean of three replica siRNA in the presence of cisplatin vs. the mean of three replica siRNA in the absence of cisplatin, and expressed as surviving fractions, as previously described [50]. Median centered Z-scores were calculated from the surviving fractions, in order to allow comparisons.

Validation of HTS screen

Two distinct siRNA sequences targeting each candidate hit kinase were used to revalidate results from the screen (against *FGFR2*: siRNA IDs 118292 and 1215; against *PRKCD* siRNA IDs 773 and 775; against *PRKCB1* siRNA IDs 103309 and 261007 against *PRKCZ* siRNAs ID 103575 and 103679, Ambion). A significance threshold of $p<0.05$ (One-way ANOVA) was expected for each siRNA. Validation of RNAi gene silencing was evaluated 48 h after transfection by Western blotting for protein expression and by quantitative RT-PCR (Taqman) for mRNA expression.

Antibodies and Western blotting

Protein expression was analyzed by immunoblotting as described in [51]. The following antibodies were used: anti-Bek-1, anti-PKCδ, anti-PKCβ1, anti-PKCζ, anti-Caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA USA), anti-phosphoThr-PKCδ, anti-Puma, anti-Bcl-X_L, anti-Bad (Cell Signalling Technology, Danvers, MA, USA), anti-Mcl-1, anti-BCL2, anti-Bim, anti-Bid, anti-Bak, anti-Bax (Epitomics, Burlingame, CA, USA) and anti-β-actin (Sigma).

Quantitative RT-PCR

Total RNA was extracted using the RNAeasy kit (Qiagen, Basel Switzerland) and converted into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen) for RT-PCR according to manufacturer's instructions. Real time qPCR was performed using

Assays-on-Demand Gene Expression products (Applied Biosystems) FGFR2 (Hs01552926_m1), PRKCD (Hs00178914), and PRKCZ (Hs00177051_m1), PRKCB1 (Hs01034075), BCL2 (Hs00153350_m1) and GAPDH (Hs99999905_m1). Relative mRNA expression levels were calculated using the comparative threshold cycle (CT) method [52].

Cell viability assay

Neuroblastoma cell lines (3 x 10^3 cells/well) were seeded in 96-well plates and grown for 72h in 10% FCS-containing medium in the presence of inhibitors, growth factors or vehicle. Cell proliferation was assessed by MTS assay using the CellTiter 961 Aqueous One Solution Cell proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Fluorescence-Activated Cell Sorting Analysis

For the assessment of apoptosis, cells were analyzed by quantitative analysis of phosphatidylserine exposure as previously described [53]. Cells were stained with Annexin V-FITC/PI following manufacturer's instructions (Biotium, Hayward, CA, USA). Apoptosis was expressed as a percentage of Annexin-V and PI positive cells.

Plasmid transfection

Stable transfections were performed with Lipofectamine Plus (Invitrogen) following the manufacturer's instructions. Cells were transfected with *PRKCD* constructs cloned into pWZL Neo Myr Flag retroviral plasmid (Plasmid 220603, Addgene, Cambridge, MA, USA) and empty vector as control. 48h after transfection, cells were diluted 1:10 in culture medium containing G418 (0.8 mg/ml, Calbiochem). A mixed population of resistant cells was expanded and analyzed after selection.

Immunohistochemistry

Paraffin-embedded tissue arrays mounted with 91 biopsies of neuroblastoma patients were obtained from the University Medical Center Hamburg-Eppendorf, Germany. For immunohistochemistry, the sections were prepared and stained as described in ref. [54], except the epitope retrieval was done by Proteinase K digestion for 5 min at 37°C. The antibodies anti-FGF-2 rabbit polyclonal antibody (ab16828) and anti-FGFR2 rabbit polyclonal antibody (ab10648) were purchased from Abcam (MA, USA).

DNA microarray analysis

Generation of tumor gene expression profiles was previously described in ref. [55] and the clinical characteristics of the neuroblastoma patients (n = 251) in ref. [56]. Raw and normalized microarray data are available at ArrayExpress (accession: E-TABM-38).

Statistical analysis

All statistical tests were performed with the software GraphPad Prism, except the CI values which were calculated with the software Calcusyn. The statistical significance of differences between groups was assessed with one way or two way ANOVA, using either Tukey or Bonferroni's multiple comparison test; $P < 0.05$ was indicated with one asterisk, $P < 0.01$ with a double asterisk, $P < 0.001$ with a triple asterisk.

2.4 Results

siRNA screen to identify kinases mediating cisplatin resistance

To identify genes conferring cisplatin resistance in neuroblastoma, we designed a robust RNAi-based loss-of function screen using a library of short interference RNA duplexes (siRNA) targeting each of the 719 known protein and lipid kinases in the human genome. The screen was performed in two well-characterized neuroblastoma cell lines (LAN-1, SH-SY5Y). The cells were systematically transfected with the siRNAs and subsequently treated with low dose cisplatin and or vehicle. The dose of cisplatin used in the screen was optimized for each cell line to achieve a sensitizing effect of 20% decrease in cell survival (EC_{20}) (data not shown). The results of the screen are represented as Z-scores approximating a normal distribution and are displayed in (**Figure** 2-1a,b). In large scale screening approaches off target effects represent a significant source of false positive hits. Redundancy in the system most likely confirms RNAi target specificity [22]. To rule out off targets effects, we only considered a gene as a candidate drug resistance gene if at least two independent siRNAs, out of the three sequences which were included in the library, caused sensitization to cisplatin scaled by an average Z-score bellow – 3. Within the candidates in LAN-1 and in SH-SY5Y (supplementary Table 1) that fulfilled these criteria, *FGFR2* (Z-score -4) resulted the top candidate in LAN-1 and *PRKCD* (Z-score -3) in SH-SY5Y. To validate the results obtained in the screen, two different individual siRNA sequences targeting FGFR2 and PRKCD were reassayed in both cell lines. As expected, resistance to cisplatin was conferred by PRKCD and FGFR2 downregulation in SH-SY5Y and LAN-1, respectively. Although not as strong as in LAN-1, the downregulation of FGFR2 also sensitized SH-SY5Y cells to cisplatin (**Figure** 2-1c).

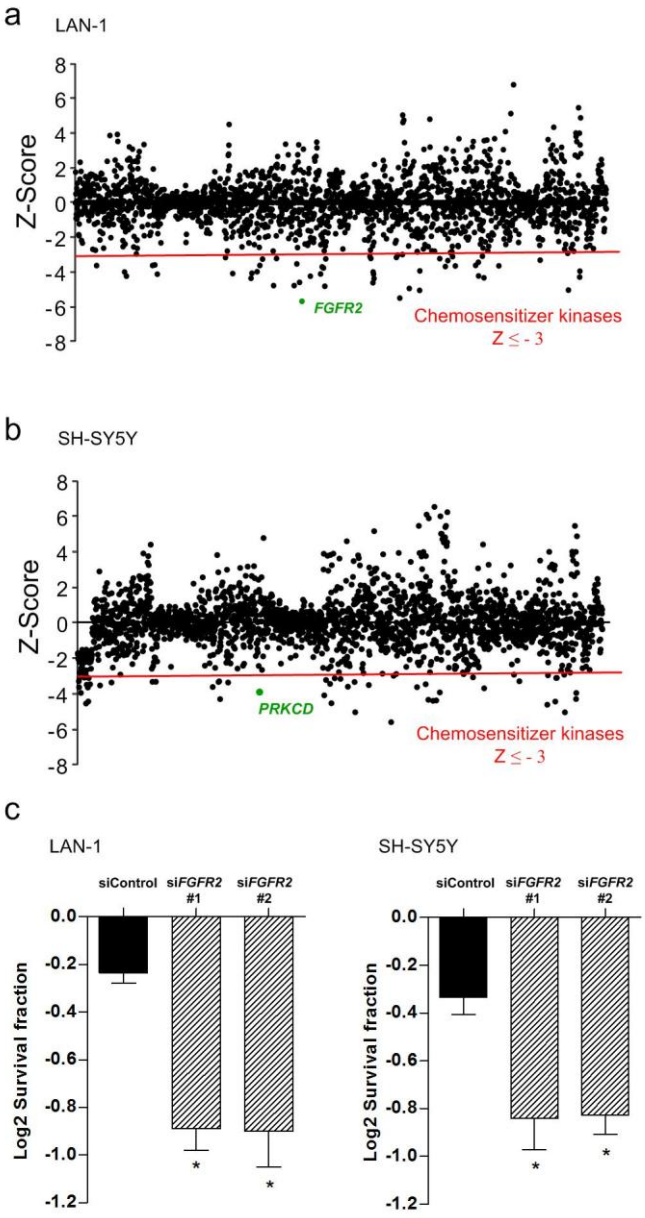


Figure 2-1 Human kinase siRNA high-throughput screen for cisplatin sensitizer kinases (a,b) Scattered plot of average Z-Scores from cisplatin sensitivity screen. (c) Cisplatin sensitivity assay repeated in triplicate for the hit candidate kinases FGFR2 with two siRNA sequences in SH-SY5Y and LAN-1 cells. Survival fractions are shown, * $P < 0.05$ compared with scrambled siRNA control, error bars represent the SD of the mean of two independent experiments.

The differences in the sensitivity to FGFR2 downregulation might be due to the lower expression levels of FGFR2 in SH-SY5Y compared to LAN-1 (Supplementary Figure 2). Thus, the role of FGFR2 as a drug resistance candidate gene was further investigated.

Validation of siRNA screen hit candidates

To confirm the specificity of the observed effects, the two different siRNA sequences targeting *FGFR2* were analyzed for target downregulation. Both siRNAs efficiently suppressed *FGFR2* expression at mRNA and protein level (Figure 2-2a), indicating that the HTS results were likely to be "on-target". In order to establish the sensitivity to cisplatin treatment, dose-reponse curves were performed (Figure 2-2b). *FGFR2* silencing significantly shifted EC_{50} values to lower cisplatin doses (siControl EC_{50} = 4.17, si*FGFR2* EC_{50}= 1.32 in LAN-1, siControl EC_{50}= 5.02, si*FGFR2* EC_{50}= 1.59 in SH-SY5Y).

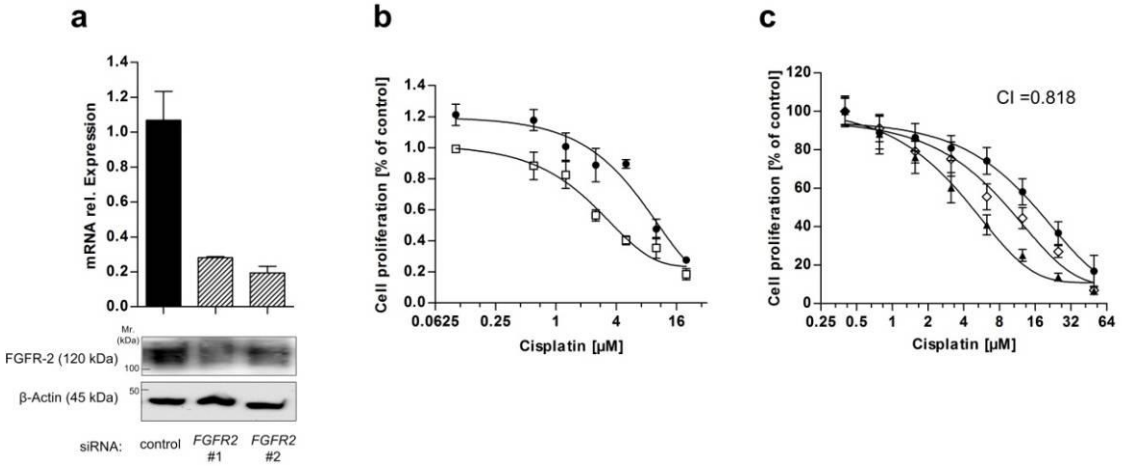


Figure 2-2 Validation of cisplatin sensitizing candidate kinases (a) Validation of the siRNA transfection efficiency. Taqman PCR and Western blot showing FGFR2 gene downregulation after 48h of transfection with two different siRNA sequences in LAN-1 cells. (b) Cisplatin titration in LAN-1 cells transfected with siFGFR2 (white squares) or control siRNA (black circles), EC50 (siFGFR2) = 1.32, EC50 (siControl) = 4.17. (c) Cell viability assay in LAN-1 cells treated with increasing concentrations of cisplatin (circles), increasing concentrations of cisplatin in presence of SU-5402 at a constant ratio of 1.25 (triangles) and increasing concentrations of SU-5402 (white squares). The combination index according to the Chou-Taladay method is CI = 0.818. Error bars represent the SD of three independent experiments.

Further confirmation that FGFR2 signalling modulates resistance to cisplatin was obtained using SU-5402, a pharmacological inhibitor against FGFR2 (Figure 2-2c). To quantify drug synergism, the

combinational index CI was calculated by the Chou-Talalay method (CI= 0.818) indicating synergism between cisplatin and SU-5402 [23]. These results provide strong evidence of the potential role of FGFR2 – signalling in neuroblastoma chemoresistance.

RNAi targeting of FGFR2 induces apoptosis in combination with cisplatin

Cisplatin induces apoptosis associated with DNA damage [24]. We initially investigated whether *FGFR2* gene silencing sensitized cells to cisplatin by triggering apoptosis. Following *FGFR2* downregulation, the percentage of apoptotic cells was increased from 23.6 to 37.9 % as observed from the percentages of Annexin V-positive/PI-negative cells (Figure 2-3a). This event was accompanied by caspase-3 activation at a low cisplatin dose, at which no sign of apoptosis manifested by caspase-3 cleavage was observed in the control cells (Figure 2-3b).

In order to investigate how FGFR2 initiates the cell death machinery, we analyzed the expression levels of the proapoptotic, as well as the antiapoptotic members of the BCL2 family of proteins (Figure 2-3c). Within the proapoptotic BH3-only proteins, only Bad was upregulated in response to cisplatin treatment, and this was only observed in cells which had *FGFR2* downregulation. Bim, Bid and Puma levels either decreased or stayed unchanged. Mcl-1 has been previously shown to modulate cisplatin induced apoptosis [25]. In neuroblastoma cells, Mcl-1 expression was not affected by cisplatin treatment. In contrast, the expression of the anti-apoptotic proteins BCL2 and BCL-X_L increased in response to cisplatin treatment. In addition, BCL2 expression correlated with the expression of FGFR2, suggesting that BCL2 expression is regulated by FGFR2 signalling. To confirm whether FGFR2 regulates BCL2 expression, the receptor was stimulated by its main ligand fibroblast growth factor-2 (FGF-2) and probed for BCL2 expression. BCL2 levels dramatically increased after 24h of growth factor stimulation in a dose dependent manner (Figure 2-3d). This effect was evident for protein expression as well as for mRNA

expression, pointing out that the regulation of BCL2 by FGFR2 occurs at least in part at the transcriptional level.

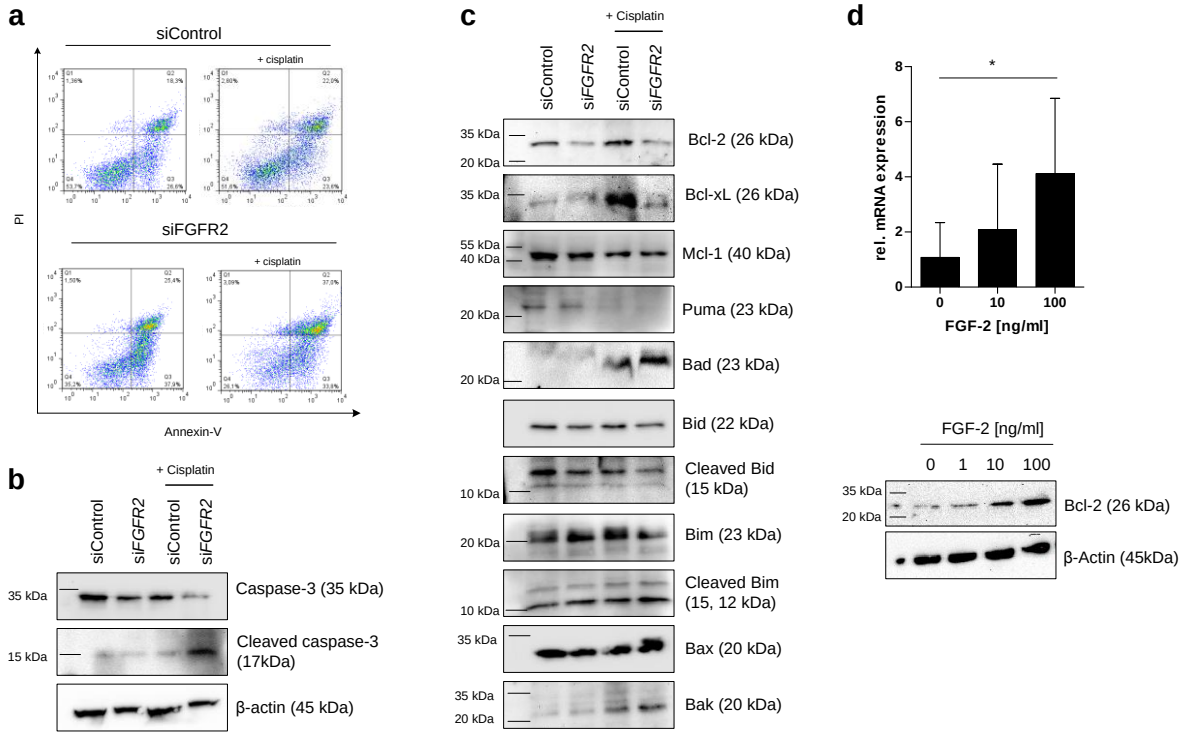


Figure 2-3 FGFR2 Silencing sensitizes neuroblastoma cells to cisplatin induced apoptosis (a) FACS plots showing annexin V and propidium iodide (PI) staining after transfection of siFGFR2 or scrambled siRNA in presence or absence of cisplatin (1.5 µM) in SH-SY5Y cells. Annexin V-positive/PI-negative cells were considered as apoptotic cells. Percentages of apoptotic cells were as follows: siControl cells, 26.6 % (- cisplatin) and 23.6 % (+ cisplatin); siFGFR2 cells 37.9 % (- cisplatin) and 33.8 % (+ cisplatin). (b) Cell lysates of siFGFR2 or siCONTROL transfected cells treated with 5µM cisplatin were analyzed by SDS Page and Western blot for apoptosis induced by caspase-3 activation. The bands corresponding to procaspase-3 (inactive), cleaved caspase-3 (active) and β-actin (loading control) are shown. (c) Western blot analysis of the indicated BCL2 family members in lysates of LAN-1 cells treated as described in (b). (d) BCL2 protein and mRNA levels quantified by taqman PCR of LAN-1 cells stimulated for 24h with increasing concentrations of FGF-2.

Protein kinase C regulates cell survival in neuroblastoma downstream of FGFR2

We next aimed at analyzing the mechanistic insights of the regulation of BCL2 expression by FGFR2. Given that FGFR2 is known to activate PKC family members through recruitment and activation of

PLCγ [26], we analyzed the possible implications of PKC in FGFR2 signalling in neuroblastoma. In the initial RNAi screen, we assessed changes in cell survival induced by the inhibition of protein and lipid kinases in presence or absence of cisplatin. Not only RNAi targeting of the protein kinase C family member PKCδ, but its isoforms PKCβ1 and PKCζ showed a role in neuroblastoma survival (Supplementary Figure 1), and were as well validated. The RNAi-mediated downregulation of all three isoforms significantly decreased cell proliferation in both cell lines, suggesting that PKC signalling may have an important role in neuroblastoma survival (Figure 2-4a). In LAN-1 cells the downregulation of *PRKCD* alone strongly inhibited cell proliferation, thus, its combination with cisplatin had rather modest effects (Figure 2-4a). Among the PKC isoforms, the inhibition of PKCδ had the strongest impact on cell proliferation in both cell lines, which was further confirmed with isoform-selective pharmacological inhibitors (Figure 2-4b). Previous studies show that PKC regulates important cell survival pathways [27]. Western blot analysis revealed that the downregulation of PKCδ attenuated important cell survival pathways such as the ERK1/2- and mTOR/S6-signalling pathway (Figure 2-4d, e). Moreover, the downregulation of *PRKCD*, but not *PRKCB1* or *PRKCZ*, induced apoptosis (Figure 2-4c) and downregulation of BCL2, suggesting its involvement in the control of apoptosis regulated by BCL2 expression (Figure 2-4d, e) and raising the hypothesis that PKCδ may act downstream of FGFR2 to regulate BCL2 expression.

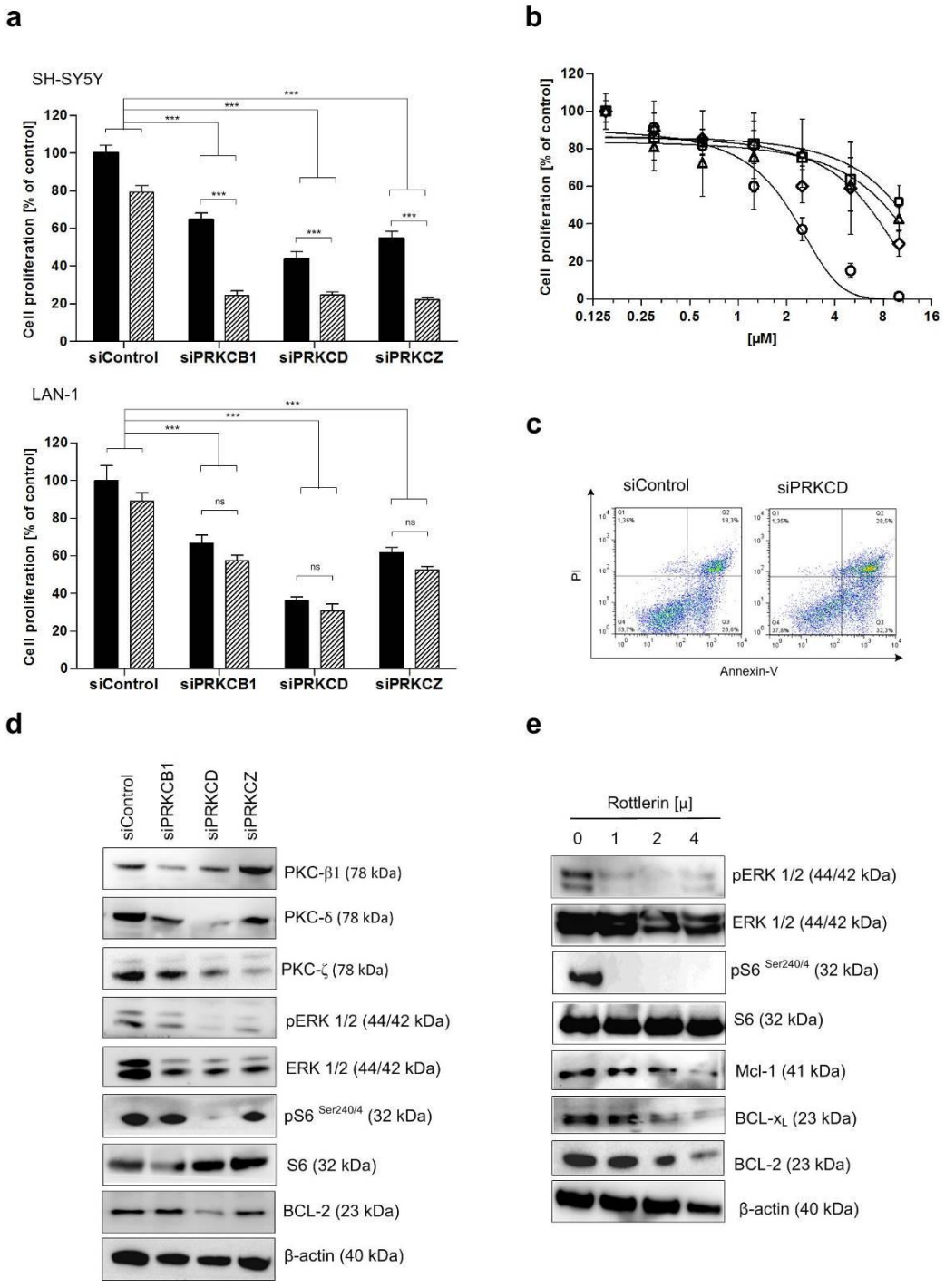


Figure 2-4 Roles of PKC family isoforms in neuroblastoma cell survival and chemoresistance (a) Cisplatin sensitivity assay repeated for PKC family members PKC-β1, PKC-δ and PKC-ζ with isoform-specific siRNA sequences in SH-SY5Y and LAN-1 cells. The effects on cell viability of the siRNA-mediated target downregulation in presence (striped bars) or absence of cisplatin (black bars) was normalized to the non-targeting siControl and expressed as percentages. (b) Cell viability assay of LAN-1 cells treated for 72h with isoform-selective PKC inhibitors;

Rottlerin (circles), PKCβ1 inhibitor (squares), PKCζ inhibitor (triangles), pan-PKC inhibitor (rhombus). (c) Apoptosis was assessed by quantification of annexin V/propidium iodide (PI) staining after transfection of siPRKCD (26.6 %) or siControl in SH-SY5Y (32.3%) cells. (d) Protein expression analysis by Western blot of LAN-1 cells transfected with siPRKCB1, siPRKCD and siPRKCZ.(e) Cell lysates of LAN-1 cells incubated with Rottlerin (PKCδ inhibitor) for 24h were analysed for protein expression and phosphorylation of the indicated proteins.

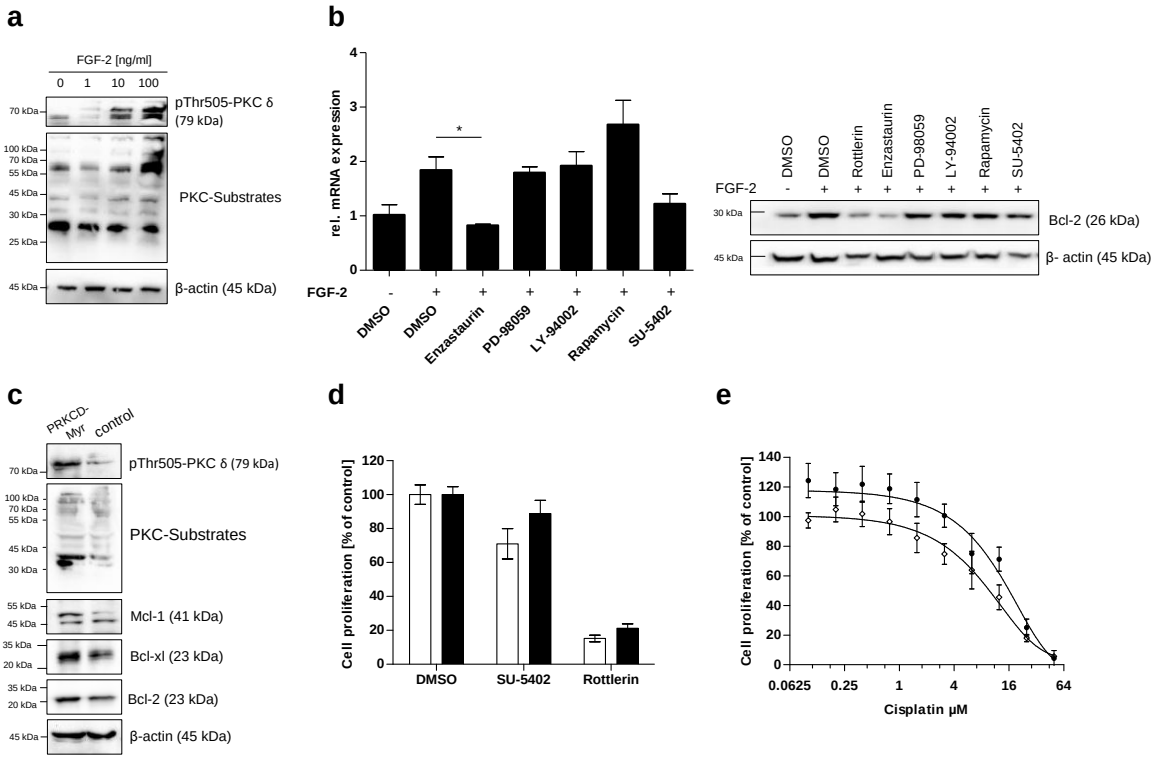


Figure 2-5 FGFR2 activates PKCδ to modulate cisplatin resistance (a) LAN-1 cells were incubated with FGF-2 for 24h. Cell lysates were analysed for PKC-activity by Western blot. (b) LAN-1 cells incubated for 24h with the inhibitors (Enzastaurin 10µM, Rottlerin 5 µM, PD-98059 25µM, LY-29402 10µM, Rapamycin 100µg/ml, SU-5402 10µM and DMSO as negative control) and 0.1µg/ml of FGF-2. BCL2 mRNA levels quantified by taqman PCR and protein levels analyzed by Western blot are shown. (c) Protein extracts of Myr-PKCδ expressing LAN-1 cells were analyzed by Western blot for activated PKCδ (pPKCδ) and p-PKC Substrates and the indicated BCL2 family members. (d) Cell viability assay of stably expressing Myr-PKCδ cells incubated for 72h in presence of FGFR2 inhibitor (SU-5402 5 µM), PKCδ inhibitor (Rottlerin 5 µM) or DMSO. (e) Cisplatin titration of stably expressing Myr-PKCδ (EC50 = 12.11) and vector transfected cells (EC50 = 8.83).

To investigate this model, we first analyzed PKCδ activation in response to FGF-2 stimulation. Activation by phosphorylation of PKCδ occurred in a dose-dependent manner in response to FGF-2 and this correlated with the phosphorylation of PKC substrates (Figure 2-5a).

FGF-2-induced BCL2 expression is mediated by PKC signalling but not MEK/ERK or PI3K/AKT/mTOR/S6K signalling

In view of our previous results, we investigated the possibility that FGF-2-mediated activation of PKCδ could account for BCL2 regulation. Inhibition of different signalling pathways downstream of FGFR2 will reveal which of them is important to regulate BCL2, if FGF-2 fails to induce BCL2 expression upon pathway inactivation. To prove this hypothesis we treated LAN-1 cells with FGF-2 and a panel of inhibitors including rottlerin and enzastaurin which target PKCδ and PKCβ1, PD-98059 a MEK inhibitor, LY-29402 a pan-PI3K inhibitor, rapamycin an mTOR inhibitor and SU-5402 which targets FGFR, and analyzed their effects on BCL2 expression. The concentrations of the inhibitors used were optimized to achieve optimal pathway inactivation (data not shown). The comparative western blot analysis reveals that only rottlerin and enzastaurin strongly inhibited FGF-2 mediated upregulation of BCL2 (Figure 2-5b). In contrast, the inhibition of the PI3K pathway or MEK/ERK pathway had no effect on BCL2 expression. SU-5402 reduced the levels of BCL2, but only modestly compared to the observed effects by rottlerin or enzastaurin.

Activation of PKCδ increases drug resistance in neuroblastoma cells

Our results had demonstrated that FGFR2 signalling uses PKCδ to activate a prosurvival mechanism involving BCL2 regulation. To further support this model, we stably transfected LAN-1 cells with a myristoylated catalytically active *PRKCD* mutant. The expression of the constitutively active PKCδ mutant was confirmed as the cells showed higher levels of PKC substrate phosphorylation compared to cells transfected with the empty expression vector (Figure 2-5c). Western blot analysis of the anti-apoptotic BCL2 family members

showed that activation of PKCδ correlated with increased Bcl-X_L and BCL2 levels, although the increases in BCL2 expression were rather modest (Figure 2-5c). We investigated whether activation of PKCδ could reverse the sensitivity of cells to FGFR2 inhibition. Therefore, we treated the stably transfected cells with the inhibitor SU-5402 and assessed cell proliferation. The *PRKCD*-Myr expressing cells were less sensitive to FGFR2 inhibition than cells transfected with the empty vector (Figure 2-5d). Inhibition of PKCδ by rottlerin lead to a significant decrease of cell survival in both transfected cell lines, but the difference in the sensitivity to PKCδ inhibition was less marked than in the case of SU-5402. Finally, cells transfected with activated PKCδ displayed enhanced survival under normal culture conditions (data not shown) and were more resistant against cisplatin treatment (EC_{50} shift from 8.83 to 12.11, Figure 2-5e). Together, these results show the ability of activated PKCδ to uncouple from FGFR2 signalling and to provide neuroblastoma cells a more chemoresistant phenotype.

Importance of FGFR2 signalling in neuroblastoma chemoresistance

To further confirm the potential role of FGFR2 in neuroblastoma chemoresistance, we used a cell line model derived from LAN-1 cells that were selected for doxorubicin resistance, and have proven as well resistance against other chemotherapeutic agents including cisplatin [28]. We analyzed the expression of FGFR2 and FGF-2 in LAN-1R cells (Figure 2-6a). FGFR2 was significantly overexpressed compared to its expression in LAN-1 cells. Moreover, LAN-1R cells expressed FGF-2 in protein and mRNA level, whereas in the other NB cell lines FGF-2 was only detected at mRNA level (Figure 2-6b). In LAN-1R the elevated levels of FGF-2 and FGFR2 were correlated BCL2 expression, which is in agreement with the previous observations, that BCL2 was overexpressed in comparison to the parental cell line [28]. These results demonstrate the importance of FGFR2 signalling in neuroblastoma acquired chemoresistance.

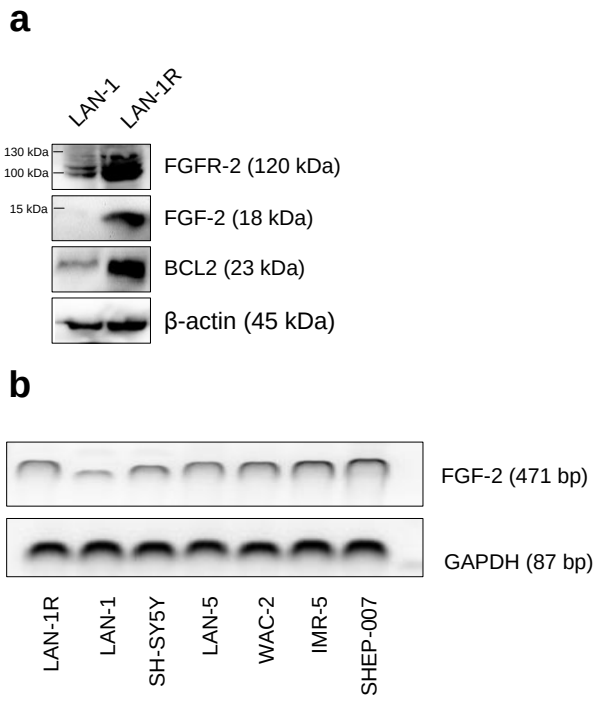


Figure 2-6 Protein expression of LAN-1R cells (a) Protein extracts of LAN-1R cells were analyzed by Western blot for FGFR2, FGF-2 and BCL2. (b) Semi quantitative PCR of FGF-2 in NB cell lines.

Targeting BCL2 inhibits cell survival and enhances the effects of cisplatin in neuroblastoma cells

ABT-737 is a potent BCL2 and Bcl-X_L inhibitor that has shown great antitumor effects in preclinical models and has entered Phase I clinical trials for the treatment of adult cancers [29]. Given that neuroblastoma cells induced BCL2 as a pro survival mechanism to overcome cell death, targeting BCL2 with pharmacological inhibitors provides a rational to efficiently induce cell death in neuroblastoma. Here, we tested the effects of ABT-737 in the neuroblastoma cell lines SH-SY5Y, LAN-1. In both cell lines, increasing concentrations of ABT-737 impaired cell survival and had a strong synergistic effect with cisplatin (CI=0.512 for SH-SY5Y and CI=0.411 for LAN-1, Figure 2-7a, b). In addition, we investigated if neuroblastoma cell lines that display a chemoresistant phenotype were also sensitive to BCL2 inhibition. We therefore investigated the sensitivity to ABT-737 in LAN-1R cells and the above described LAN-1 cells, which stably express activated PKCδ. Also in both cell lines, ABT-737 inhibited cell

survival and had synergy with cisplatin, although compared with the parental cell line LAN-1, the combinational effect with cisplatin in LAN-1R was less significant (LAN-1-PRKCD-myr CI=0.473, LAN-1R CI = 0.633, Figure 2-7c,d).

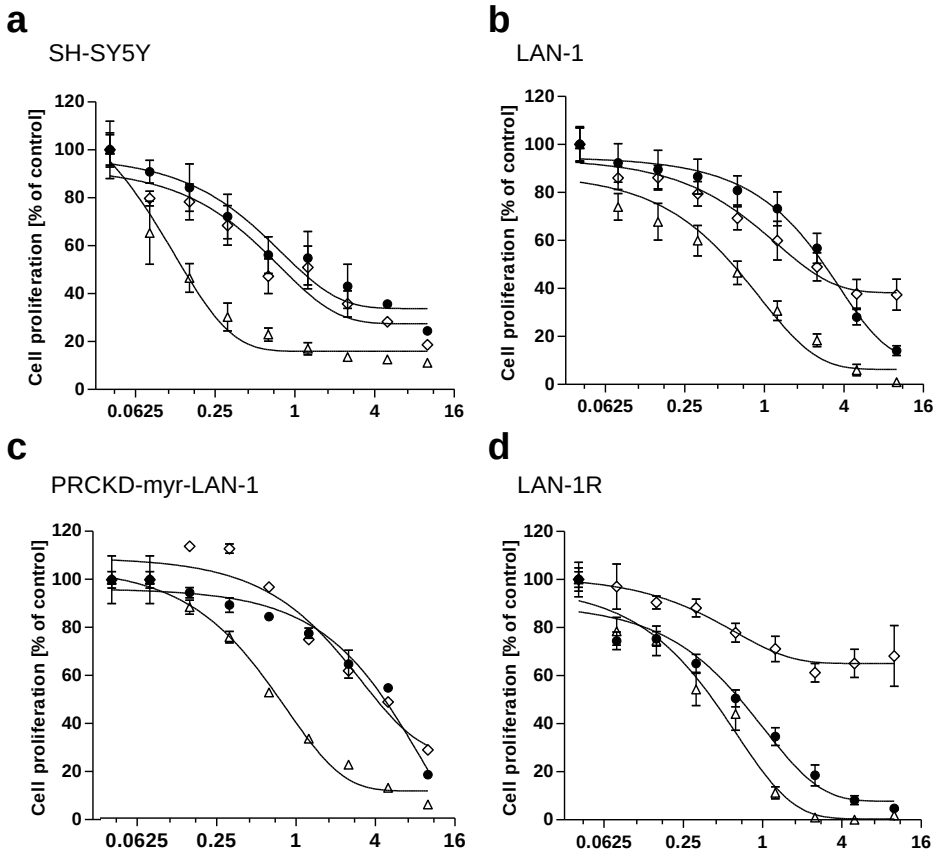


Figure 2-7 Targeting ABT-737 in neuroblastoma cell lines (a,b,c,d) Cell viability assay of SH-SY5Y (a), LAN-1 (b), PRKCD-myr LAN-1 transfectant (c) and LAN-1R (d) treated for 72h with ABT-737 (circles), cisplatin (rhombus) and the combination of ABT-737 and cisplatin at a constant ratio (1:2.5). Combinational indexes were calculated (SHSY5Y CI = 0.512; LAN-1 CI = 0.411; (PRKCD-myr LAN-1 transfectant CI = 0.473; LAN-1R CI = 0.633). Error bars represent the SD of the mean of three independent experiments.

Expression of FGFR2 in NB tumor samples

In order to investigate the clinical relevance of FGFR2 and FGF-2 in neuroblastoma tumor samples, a total of 91 samples of neuroblastomas available as a tissue microarray (TMA) were probed for protein expression by immunohistochemical staining (Figure 2-8a-d). FGFR2 was detected in all neuroblastoma primary tumors, whereas 80% was

scored as positive (53 of 66), 3% as strong positive (2 of 66) and 17% as weak positive (11 of 66). Among the metastatic tumors 81% were positive (21 of 26) and 19% were weak positive (5 of 26). Expression of FGF-2 was quantified positive for 30% of primary tumors (20 of 67), strong positive for 3% (2 of 67), weak positive for 61% (41 of 67), and negative for only 6% (4 of 67). Notably, metastatic tumors showed higher scores for FGF-2, only one tumor was negative for FGF-2 accounting for 4% of all samples of metastatic tumors (26), 42% were positive (11 of 26) and 56% were weak positive (14 of 26).

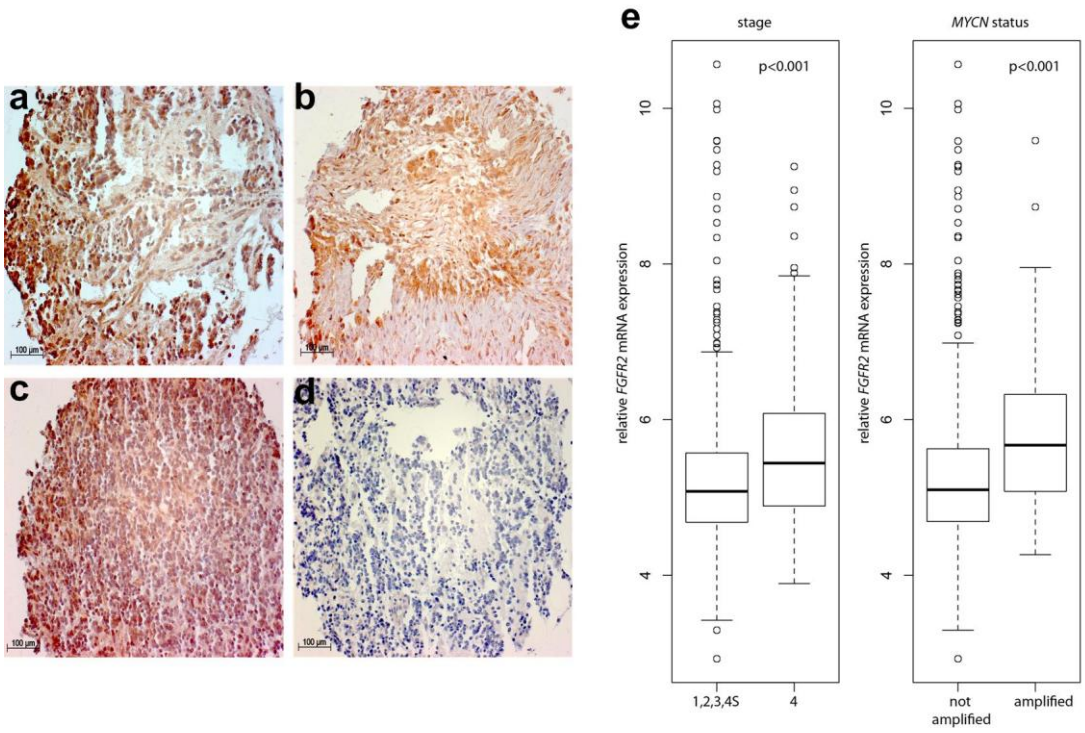


Figure 2-8 Analysis of the expression of FGFR2 and FGF-2 in NB patients (a-d). Representative NB samples of TMA analysis showing the different immunoreactivities for FGFR2 in positive (a) and weakly positive samples (b) and for FGF-2 in positive (c) and negative samples (d). (e) DNA microarray analysis of 251 NB patient samples showing the expression of FGFR2 in correlation to tumor stage (stages 1,2,3,4S vs. stage 4, left panel) or to *MYCN* amplification status (right panel). P values from the Wilcoxon-test are indicated.

Consisting with these findings, a DNA microarray study of 251 patient samples revealed that FGFR2 is expressed in neuroblastoma tumors and it moreover correlates with higher disease stage and *MYCN* amplification (Figure 2-8e).

2.5 Discussion

The poor outcome of high-risk neuroblastoma patients with current multimodal therapies justifies the development of novel targeted therapies for this common childhood cancer. The development of chemoresistance has been demonstrated to be a major cause for treatment failure in neuroblastoma, as well as in many other human cancers [1, 17]. Cisplatin is commonly used in current multimodal therapeutic regimens in high risk NB and multiple resistance mechanisms have been described against this drug in human cancer [2-4]. It has been demonstrated that the deregulation of intracellular signalling pathways controlling apoptosis plays a major role in cisplatin resistance in human cancer [2]. By using high throughput RNAi screens in established NB cell lines, we have identified novel potential drug targets involved in NB survival and resistance to cisplatin. A previous report using RNAi screening identified novel protein kinases involved in NB cell proliferation [30]. Amongst these kinases, CHK1 was validated as a novel target [30]. In our screen, the downregulation of CHK1 did not show significant effects in cell survival, the different results might be explained due to the biological variability within the cell lines used. However, in agreement with this screen, we identified in our cell lines also BMPR1A and RPS6KB1 as hit candidate kinases. Moreover, our screen identified AURKB as an important regulator of cell survival in neuroblastoma cells harboring *MYCN* amplification, consistent with a previous study in which the related isoform AURKA was identified from a shRNA screen as a gene that is required for the growth of *MYCN*-amplified neuroblastoma cells [31].

The comparative analysis of the kinases identified as chemosensitizer kinases in the NB cell lines used in our study revealed that FGFR2 was the most promising candidate. LAN-1 cells have *MYCN* amplification, while SH-SY5Y are *MYCN* single copy. This may in part explain that there were significant differences in the identity of the chemosensitizer kinases identified in the two NB cell lines. Intriguingly, *MYCN*

expression also correlated with FGFR2 expression in primary NB, which may account for the stronger impact of FGFR2 silencing on cisplatin sensitivity in LAN-1, as compared to SH-SY5Y. In support of our findings, the FGFR2 has been recently implicated in modulating resistance to cisplatin kinase in ovarian cancer [32].

Alterations in *FGFR2* have been widely identified in human cancers. Gene amplification or mutations of *FGFR2* were reported in breast cancer, gastric cancer, lung cancer, ovarian cancer, and endometrial cancer [33-36]. Genetic alterations may induce aberrant FGFR2 signalling activation due to the release of FGFR2 from auto inhibition, or through creation of FGF signalling autocrine loops [26]. In neuroblastoma, FGF-2 was previously identified in advanced-stage tumors (stages 3 and 4) compared with low-stage tumors (stages 1, 2, and 4S) [37]. Consistent with this study, our data indicate that FGFR2 is activated by an autocrine loop involving FGF2 in NB cells. This model is based on the following observations: (i) FGF-2 is consistently expressed in primary NB and NB cell lines; (ii) FGF-2 activates FGFR2 signalling in NB cell lines at nanomolar concentrations; (iii) pharmacological inhibition of FGFR2 tyrosine kinase activity blocks the effect of FGF-2 on BCL2 expression; (iv) the expression of FGF-2 and FGFR2 is up-regulated in NB cell lines with acquired chemoresistance. The observation that the expression of FGF-2 and FGFR2 is up-regulated in NB cell lines with acquired chemoresistance points to a more general role of this signalling pathway in the resistance of NB cells to other chemotherapeutic agents, such as doxorubicin. Moreover, the fact that FGFR2 is over-expressed in higher stage tumors emphasizes the importance of FGFR2 signalling in the development of malignant transformation in neuroblastoma.

Mechanistically, FGFR2 signalling altered the expression profile of anti-apoptotic BCL2 family proteins in NB cells, resulting in a decrease in the sensitivity of the cells to cisplatin-mediated apoptosis. PKC isoforms were shown to play a major role in FGFR2 signalling to BCL2, in contrast to other signalling pathways, such as PI3K/mTOR

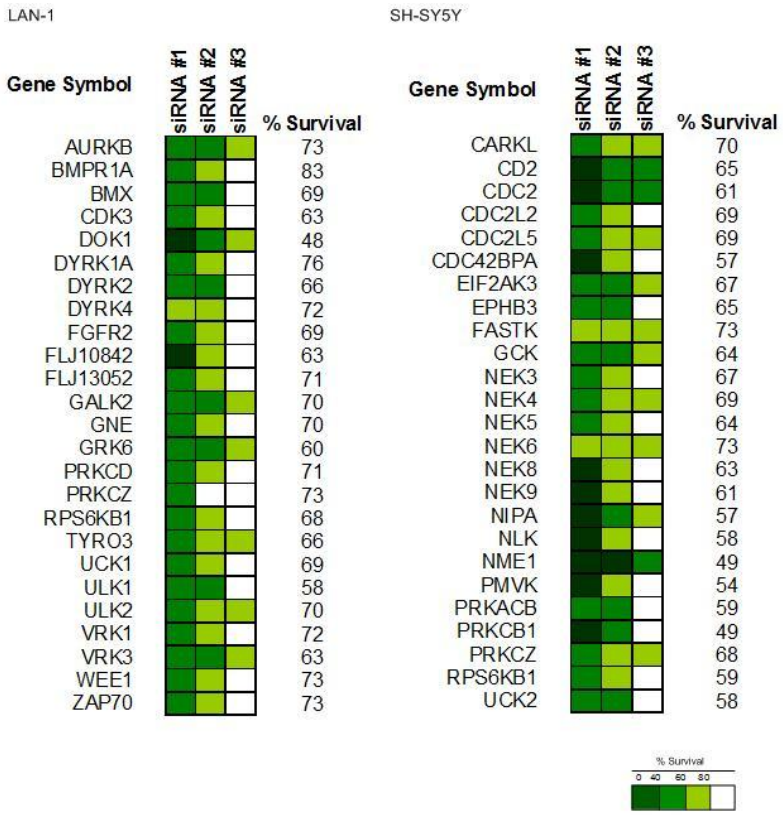
and Erk. BCL2 family proteins have been extensively studied for their role in modulating cancer chemoresistance. A previous study has also documented the potential of targeting BCL2 family proteins with ABT-737 in NB [38, 39], which is consistent with our findings. Previous reports have documented that FGF-2 signalling protects small cell lung cancer (SCLC) cells from chemotherapy-induced apoptosis by modulating the expression levels of BCL2 family proteins [40, 41]. Intriguingly, PKC isoforms were reported to be involved in transducing FGF-2/FGFR-1 signals to BCL2 family proteins in SCLC cells, but the mechanism involved post-transcriptional events [40]. In contrast, FGF-2-mediated up-regulation of BCL2 occurred at the transcriptional level in NB cells, which may be accounted for by differences in FGFR isoform involvement. Several transcription factors may be involved in transducing the signal from FGFR2/PKC to BCL2, including NF-kB [42]. PKC is known to regulate the inhibitor of kappa B kinase (IKK) and induce NF-kB activation B- and T-cells, a process which is important for the adaptive immune response [43, 44], although the main role is attributed to the isoform PKC-β1, it has been as well reported that NF-kB is activated by PLC-γ and PKCδ [45]. Importantly, our data have a direct relevance to translational cancer research in NB, since there are several pharmacological inhibitors of FGFR in clinical trials, including ZD-4547, BGJ398 and FP-1039, which are entering Phase I or II for the treatment breast cancer or gastric cancers with *FGFR2* amplification, endometrial cancers with *FGFR2* mutations or advanced solid cancers with *FGFR1,2,3* amplifications [46]. In addition, Enzastaurin is also in clinical trials for other human cancers [46, 47], such as glioblastoma, multiple myeloma, non-hodgkin's lymphoma, breast cancer and non small cell lung cancer. Among them, enzastaurin has already proven antitumor effects in myeloma and NSCLC patients [48, 49]. The ability to detect FGF-2 in the urine of cancer patients may also be relevant for the future development of novel prognostic biomarkers in NB patients.

Together, our findings indicate that FGFR2 is a determinant for cisplatin response and suggest a therapeutic option to improve the efficacy of chemotherapeutic agents in the treatment of high risk neuroblastoma patients.

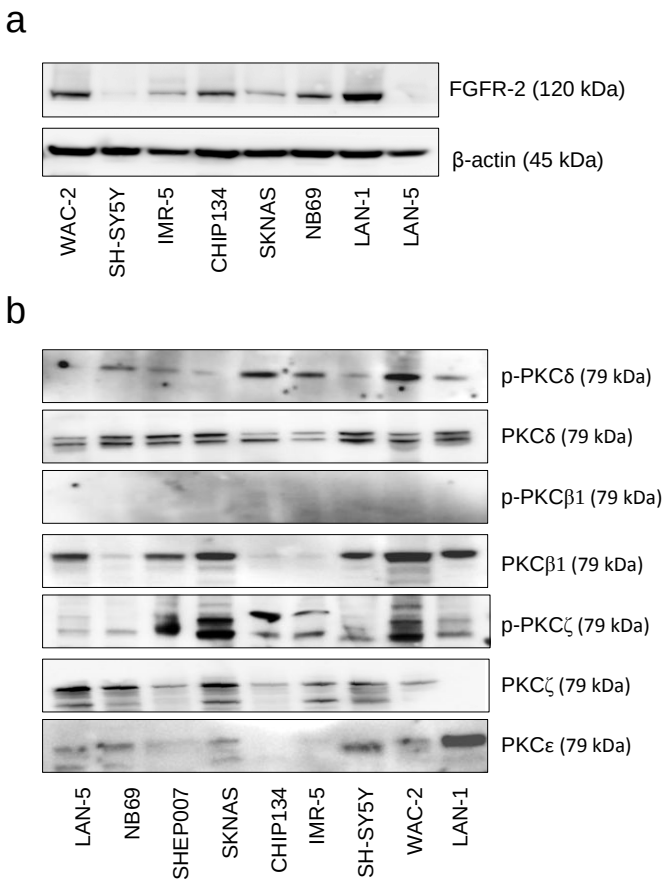
2.6 Supplementary information

SH-SY5Y		LAN-1	
Gene	Z	Gene	Z
PRKCD	-3	*FGFR2*	-4
EPHA5	-3	*RPS6KA2*	-4
EPHB1	-3	*SPHK2*	-4
FLJ10842	-3	*HCK*	-3
GALK2	-3	*WEE1*	-3
GRK6	-3	*BMPR2*	-2
MAP3K7IP2	-3	*FYN*	-2

Suppl. Table 2-1 Human kinase siRNA high-throughput screen for cisplatin sensitizer kinases. The kinases, which being down-regulated led to a sensitization to cisplatin treatment, with at least two unique siRNA sequences are listed. Z-scores are averages of the two siRNA unique sequences, which caused the sensitization to cisplatin.



Suppl. Figure 2-1 Human kinase siRNA high-throughput screen for kinases involved in proliferation. Kinases, which when being downregulated led to a > 25% decrease in cell proliferation compared with a negative control, with at least two unique siRNA sequences.



Suppl. Figure 2-2 Protein expression of NB cell lines. Whole cell lysates of NB cell lines were extracted and analysed by western blotting for the expression of the indicated proteins.

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CHAPTER 3

THE PHOSPHOINOSITIDE 3-KINASE P110A ISOFORM REGULATES LEUKEMIA INHIBITORY FACTOR RECEPTOR EXPRESSION TO PROMOTE CELL PROLIFERATION AND SURVIVAL IN MEDULLOBLASTOMA

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Manuscript in preparation.

In this manuscript, I performed all the experiments except those performed with the inducible UW228-MycER clones in figure 3.

3.1 Abstract

Medulloblastoma is the most common malignant brain tumour in children and is associated with a poor outcome. We were interested in gaining further insight into the mechanisms underlying the role of the phosphoinositide 3-kinase (PI3K) p110α isoform in medulloblastoma cell proliferation and survival. The impact of RNA interference (RNAi)-mediated silencing of p110α on global gene expression was investigated in medulloblastoma cell lines, in order to identify potential target genes. DNA microarray analysis revealed a subset of genes that were selectively altered in expression upon p110α silencing, in comparison to silencing of the closely related p110δ isoform. Amongst these genes, the leukemia inhibitory factor receptor α (LIFRα) was validated as a novel p110α target in medulloblastoma. A bioinformatics analysis revealed a gene network involving c-Myc, which controls LIFRα expression downstream of p110α. LIFRα expression was found elevated in primary medulloblastoma, in comparison to normal cerebellum. In addition, an autocrine signalling loop involving leukemia inhibitory factor and the LIFRα was uncovered in medulloblastoma cell lines. Targeting the LIFRα by RNAi or by using a neutralizing antibody impaired medulloblastoma cell proliferation and induced apoptosis. Together these data reveal a novel function of the LIFR as a downstream target of PI3K signalling in medulloblastoma cell proliferation and survival.

3.2 Introduction

Medulloblastoma is the most common malignant brain tumour in children and accounts for approximately 20% to 25% of all pediatric central nervous system tumours [1]. Most medulloblastoma occur at between 5 and 10 years of age [1, 2]. The standard treatment of medulloblastoma involves surgery followed by chemotherapy and, in the case of children older than 3-5 years, radiotherapy [3, 4]. Although these therapeutic approaches are often effective at shrinking the primary tumour, recurrence and metastasis frequently occur, so that the 5-year survival rate is below 50% for high-risk patients [3]. There is an urgent need to develop novel targeted therapeutic approaches for medulloblastoma, which will originate from a better understanding of the disease biology.

Medulloblastoma has been recognized to be a heterogeneous disease, and no recurrent cancer gene mutations have been found [4], although many of the mutations described so far affect key intracellular signalling pathways, such as sonic hedgehog (SHH) and Wnt/β-catenin [4]. Genomic studies in large series of primary medulloblastoma have led to a novel classification of medulloblastoma into different molecular subtypes, characterized by the activation of sonic hedgehog (SHH), Wnt/ β-catenin or c-Myc pathways [5, 6]. c-Myc plays an important role in medulloblastoma biology and targeting the signalling networks controlled by c-Myc may be a promising approach to develop targeted therapies for the subsets of tumors in which c-Myc is activated [7-9].

Another signalling pathway which is being considered to develop targeted therapies for medulloblastoma, is the receptor tyrosine kinase (RTK) cascade, which links polypeptide growth factors to cellular responses via their downstream signalling intermediates, in particular phosphoinositide 3-kinase (PI3K), Akt, the mammalian target of rapamycin (mTOR) and mitogen-activated extracellular signal-

regulated kinase activating kinase (MEK) [10-14]. Clinical trials have started to evaluate the safety and efficacy of agents targeting this pathway in medulloblastoma.

The PI3K signalling pathway controls key cellular responses, such as cell growth and proliferation, survival, migration and metabolism [15, 16]. Over the last decades, it has been recognized that this intracellular signalling pathway is frequently activated by genetic and epigenetic alterations in human cancer, including malignant brain tumors [6, 14]. The PI3K family of signalling enzymes comprises eight catalytic isoforms, which are subdivided into three classes. The class I_A PI3K isoform p110α is considered to be a validated drug target in human cancer, in particular because activating mutations in the *PIK3CA* frequently occur in human cancer. In medulloblastoma, the *PIK3CA* gene is targeted by mutations at a low frequency [17], but the p110α isoform is found over-expressed in primary tumors and cell lines [14]. Targeting p110α by RNAi or isoform-specific inhibitors impaired medulloblastoma cell proliferation, survival and chemoresistance [14].

In this report we have analyzed the downstream targets of p110α in medulloblastoma and show for the first time that selective gene networks, comprising c-Myc and the leukemia inhibitory factor receptor α (LIFRα) contribute to oncogenic p110α signalling in medulloblastoma.

3.3 Materials and Methods

Cell culture and treatments

The MB cell lines were obtained and cultured as described in [14], the stable clones DAOY V11 (empty vector transfected) and DAOY M2 (MYC vector transfected) were described in [18] and the c-Myc-inducible clones UW228 were described in [19]. Tetracycline and tamoxifen (Sigma, Buchs, Switzerland) were used to induce c-Myc expression.

The PI3K inhibitors PIK75 and YM024 were dissolved in DMSO at 10 mM and diluted to the indicated concentrations in cell culture medium just before use. The Anti-human LIFRα Antibody was purchased from R&D Systems (Minneapolis, MN, USA) and was diluted directly into the medium immediately before use. For growth factor stimulations, cells were grown to confluence, starved overnight in culture medium containing 1% FCS. Cells were maintained in serum-free RPMI for 1 h and were then stimulated with Oncostatin M (OSM, Sigma, Buchs, Switzerland) for 10 min.

RNA Interference

MB cell lines were transfected with siRNA pools, each comprising four individual oligonucleotides (SMARTpool small interfering RNA reagents; Dharmacon, Waltham, MA, USA), directed against *PIK3CA*, *PIK3CD*, *MYC*, *LIFR* using Lipofectamine 2000 (Invitrogen) as directed by the manufacturer for adherent cell lines. siCONTROL Non-targeting siRNA Pool (Dharmacon) composed of four siCONTROL Non-targeting siRNAs was used as negative control. All siRNAs were used at a final concentration of 20 nM. Cells were incubated for 24 to 72 h to allow target down-regulation. After 24, 48, and 72 h, mRNA and protein were extracted to assess protein expression by Western blotting and mRNA expression by TaqMan assay.

DNA microarray expression profiling and data analysis

The cDNA microarray analysis was performed in collaboration with the Functional Genomic Center of the University Zurich, Switzerland. Human Genome-U133 Plus 2.0 Affymetrix GeneChips arrays (Affymetrix, Santa Clara, CA, USA) were used to assess the gene expression data. Raw data generated by the GCOS Software (Affymetrix) were processed by using the RMA method and further statistically analyzed by using the software R and applying Student's t-test.

Each experiment represented a group of three independent biological replicates. Results are expressed as fold change, and differences in expression were considered significant if the fold change was > 2.0 or <-2 and the P-value < 0.01. Comparative and cluster analysis of the data were performed using the softwares Venny, Cluster 3.0 and TreeView. The GeneGO MetaCore (GeneGO, St Joseph, MI, USA) was used to identify affected pathways and networks of genes, according to their ontological categories.

Gene expression analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Basel, Switzerland) and converted into cDNA using High-Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Assays-on-Demand Gene Expression products (Applied Biosystems) were used to measure mRNA expression levels of *PIK3CA* (Hs00907965_m1), *PIK3CD* (Hs00192399_m1), *MYC* (Hs00153408_m1), *LIFR* (Hs00158730_m1) and *GAPDH* (Hs99999905_m1; internal control gene). Relative mRNA expression levels were calculated using the comparative threshold cycle (CT) method.

For semi-quantitative analysis of the gene expression, the One-Step RT-PCR Kit (Qiagen) was employed. The following primers were used: sequences LIF, LIFR, GAPDH (Microsynth, Balgach

Switzerland). DNA was stained with GelRed (Biotium, Hayward, CA, USA), and separated in 2.5% agarose gels.

Western blotting

Cell lysates were prepared in RIPA buffer (50mM Tris-Cl, pH 6.8, 100mM NaCl, 1% Triton X-100, 0.1% SDS) supplemented with Complete Mini Protease Inhibitor Cocktail (Roche Applied Sciences) and with the phosphatase inhibitors β-glycerophosphate (20mM) and Na3VO4 (200 mM). Proteins were separated by SDS–polyacrylamide gel electrophoresis and western blotting on polyvinylidene fluoride membranes PVDF (Amersham, GE Healthcare, UK). Antibodies specific for PI3K p110α, p110δ, c-Myc, LIFR, Akt1/2/3, ERK 1/2, STAT3, S6 protein (Santa Cruz Biotechnology, CA, USA), phospho-ERK1/2 (Thr202/Tyr204), phospho-Akt (Ser473; Thr308), phospho-S6 (Ser235/Ser236; Ser240/Ser244), phospho-STAT3 (Tyr705) (Cell Signalling Technology, Inc., Danvers, MA, USA), β-actin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Chemiluminescence was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Plasmid transfections

DAOY cells were transfected with expression plasmids using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's recommended protocol. The plasmids pcDNA3-*PIK3CA* Nr. 3 and 4 encoding wild type PI3K-p110α and pcDNA3 (negative control) were described in [20].

Cell proliferation and apoptosis assays

Cell proliferation was assessed using the Cell Titer 96 Aqueous Cell Proliferation Assay (Promega, Madison, WI, USA). Apoptosis was assessed measuring the activity of caspases 3 and 7 using Caspase-Glo 3/7 Assay (Promega) as per manufacturer’s instructions. Data are expressed as average values from three independent experiments.

Statistical analysis

All experiments were performed at least in triplicates. Data are represented as mean ± s.d. The statistical significance of differences between groups was assessed with ANOVA using Tukey's post tests using the statistical software GraphPad PRISM 5; $P < 0.05$ were considered significant and indicated with a single asterisk, a double asterisk if $P < 0.001$ or a triple asterisk if $P < 0.001$.

3.4 Results

Gene expression analysis reveals selective gene subsets regulated by p110α

In order to investigate whether the class IA PI3K isoform p110α controls the expression of specific gene subsets in medulloblastoma, we performed DNA microarray analysis in DAOY cells transiently transfected with either control siRNA, siRNA targeting p110α, siRNA targeting p110δ, or the combination of p110α and p110δ siRNAs. The efficacy of the down-regulation of the target proteins by the respective siRNAs was demonstrated by Western blot analysis, as well as quantitative RT-PCR (Figure 3-1a). Silencing p110α significantly affected the expression of over 350 genes, while silencing p110δ resulted in significant changes in about 250 genes, and both p110α and p110δ about 300. A comparative analysis revealed that 60 genes were commonly found as downstream targets of p110α and p110δ. The resulting sets of differentially expressed genes were further analyzed by using different bioinformatics tools, in order to search for functional networks between genes based on the gene ontology and to narrow down the amount of data generated (Figure 3-1).

Silencing of p110α perturbs multiple transcriptional networks in medulloblastoma

To gain insight into the transcriptional networks affected by silencing of p110α and p110δ in medulloblastoma cells, we performed a biostatistics analysis of the gene expression data with GeneGO MetaCore. The transcriptional networks that were most significantly altered comprised c-Myc, the estrogen receptor (ER), p53 and STAT3 (Table 1). The transcriptional network involving c-Myc included the LIFRα as a target gene (Figure 3-1c). LIFRα was found to be more significantly down-regulated in DAOY cells transfected with p110α siRNA, than in the case of p110δ siRNA (Figure 3-1b and Table 2), suggesting that it is selectively regulated by p110α. Interestingly,

STAT3, a downstream target of the LIFR (Figure 3-5) was identified as one of the most significantly altered networks (Table 1). Thus, down-regulation of *PIK3CA* and *PIK3CD* perturbs multiple transcriptional networks in medulloblastoma cells, most notably c-Myc, which is involved in controlling LIFRα expression [21].

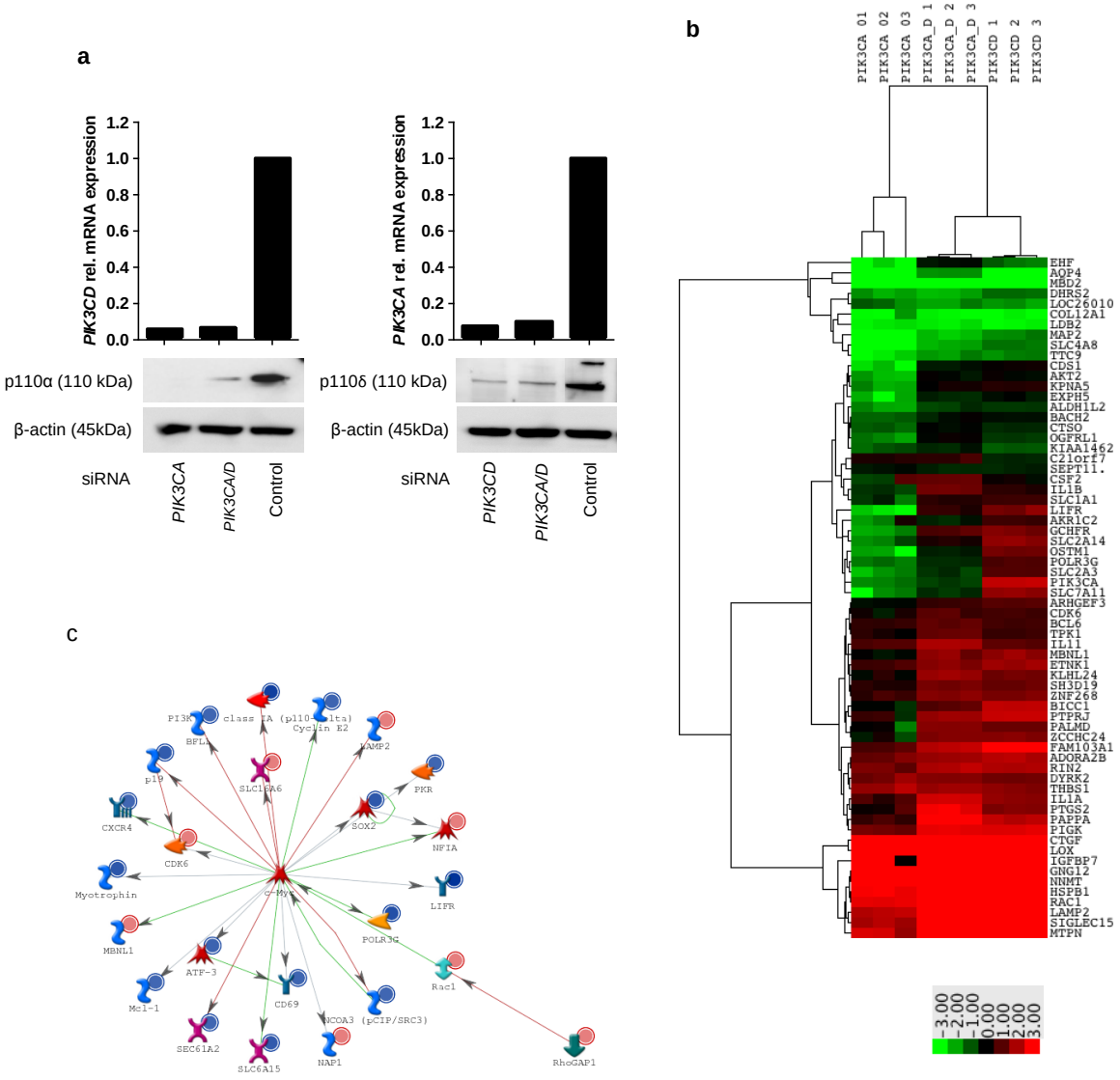

Figure 3-1 Gene expression analysis. (a) DAOY cells were transfected with siRNA against PIK3CA, PIK3CD or PIK3CA and PIK3CD and analyzed 48h post transfection for gene down-regulation at mRNA level by real-time PCR and at protein level by Western blot. (b) Heat map representing the expression of PIK3CA and PIK3CD commonly regulated genes. (c) GeneGo Metacore analysis of transcriptional networks, the network of c-Myc-regulated genes is shown.

	PIK3CA	***PIK3CD***	***PIK3CA/D***
LIFR	-6.5	-2.0	-1.3
myotrophin	-4.2	-2.6	-1.1
p19	-3.1	-2.1	-3.0
SOX2	-2.3	-4.0	-2.2
SLC6A15	-2.3	-3.2	-3.8
SEC61A2	-2.2	-3.0	-2.7
ATF3	-1.2	-2.4	-3.2
MCL1	-1.3	-2.9	-1.7
CXCR4	-1.9	-2.9	-1.8
Cyclin E2	-3.1	-1.3	1.3
CD69	-2.7	-2.3	-3.1
pCIP/SRC3	-2.7	-1.9	-2.0
BFL1	-1.7	-1.7	-3.0
POLR3G	-4.9	1.4	1.3
SLC16A6	-1.1	3.2	1.8
NFIA	1.3	2.2	2.6
MBNL1	2.0	3.2	3.7
LAMP2	2.8	5.0	6.2
CDK6	4.6	4.5	3.8

Table 3-1 Genes involved in the transcriptional network of c-Myc. Foldchanges in the gene expression of DAOY cells with were transfected with the indicated siRNAs (targeting *PIK3CA*, *PIK3CD* or both) versus control siRNA are listed for the genes which are transcriptionally regulated by cMyc.

PI3K-p110α dependent regulation of LIFRα expression

In order to validate the LIFRα as a bona fide target of p110α in medulloblastoma cell lines, we first used quantitative RT-PCR (TaqMan) and Western blot analysis to confirm the results obtained from the DNA microarrays. Down-regulation of the expression of the LIFRα could indeed be demonstrated at the mRNA and protein level upon p110α silencing (Figure 3-2a). We next sought to investigate whether pharmacological inhibition of p110α also reduced LIFRα expression levels in medulloblastoma cell lines. YM024, a selective p110α inhibitor effectively reduced the expression levels of the LIFRα in a dose-dependent manner (Figure 3-2b). The levels of LIFRα down-regulation achieved by treatment of DAOY with a low dose (1 μM) of

YM024 were comparable to the response observed upon p110α silencing by siRNA (Figure 3-2a,b).

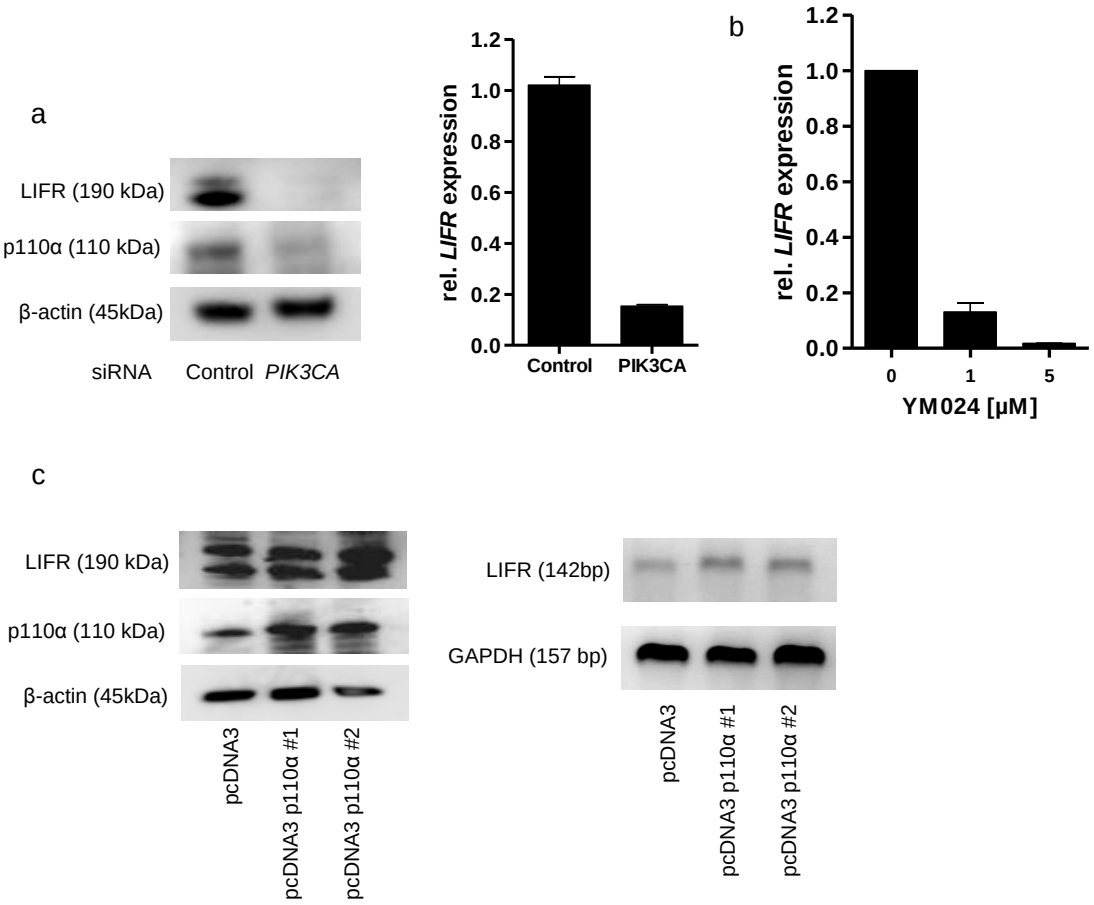

Figure 3-2 PI3K-p110α dependent regulation of LIFRα expression. Expression of LIFRα was analyzed by quantitative real-time PCR and Western blot in DAOY cells transfected with siRNA against PIK3CA (a), or treated with YM024 at 1 µM or 5 µM (b). (c) The pcDNA3 vector containing a coding sequence for PIK3CA was transfected into DAOY cells, 48h post transfection cell lysates were analyzed for PIK3CA and LIFRα protein up-regulation by Western blot or LIFRα mRNA expression semi quantitative RT-PCR.

To further confirm these observations, DAOY cells were transiently transfected with a plasmid encoding wild type p110α. Increased expression of p110α induced an up-regulation of the LIFR both at the mRNA and protein level (Figure 3-2c). Collectively, these results confirm that p110α controls the expression of the LIFRα in medulloblastoma cell lines.

Regulation of LIFR by c-Myc downstream of p110α

We next aimed at analyzing the mechanism in which p110α may regulate LIFRα expression. According to the analysis of transcriptional networks performed with GeneGo, c-Myc is a transcription factor for LIFRα. To confirm this, possible binding sites of c-Myc on LIFR promoter sequence were investigated using the software Genomatix. More than 40 possibilities were found (data not shown), providing us a hint that also in medulloblastoma cells c-Myc may bind to the LIFRα promoter.

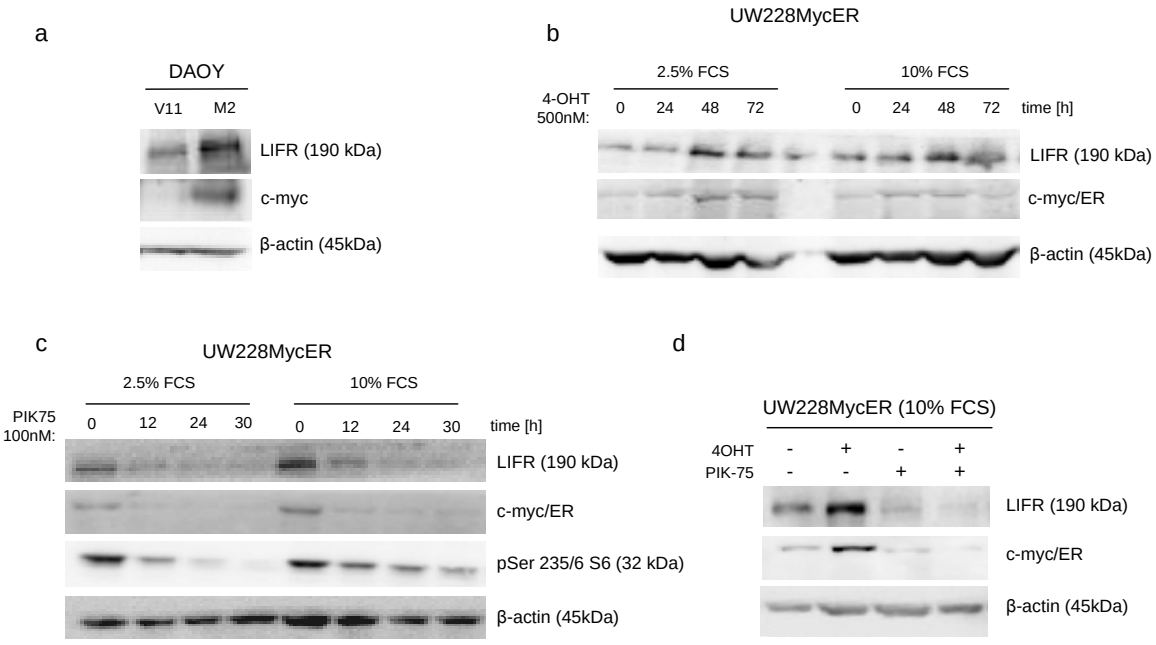

Figure 3-3 p110α regulates LIFR expression through c-Myc. The protein expression levels of c-Myc and LIFRα were evaluated by western blot in (a) DAOY-derived cell clones (empty-vector-transfected DAOY V11, MYC-transfected DAOY M14) and (b) in UW228 MycER cells induced for c-Myc expression by tamoxifen for 24h, 48h and 72h. (c) LIFRα protein level was analysed by western blot in inducible UW228 MycER cells treated with 100nM of the p110α inhibitor PIK75 for 12h, 24h, and 30h. Phospho S6 Ser 235/236 and c-Myc expression were also analysed. (d) C-Myc was induced with tamoxifen in presence or absence of 100nM PIK75 and analysed for LIFR expression.

First, we analyzed the expression of LIFRα in a DAOY-derived cell clone, which over-expresses c-Myc and we found a correlation between c-Myc and LIFR expression (Figure 3-3a). Secondly, we used the inducible MB cell line UW228-MycER which expresses a fusion

product of c-Myc and the estrogen receptor upon induction by tamoxifen. We observed that LIFR is expressed in a time dependent manner which is synchronized with the induction of c-Myc expression (Figure 3-3b). Moreover, inhibition of p110α by PIK75 abolished c-Myc induced expression of LIFRα (Figure 3-3c).

Expression of LIFRα and LIF in medulloblastoma

Our previous work has documented an over-expression of p110α in primary medulloblastoma, as well as in medulloblastoma cell lines [14]. Therefore, we hypothesized that the LIFRα may also be over-expressed in medulloblastoma. A panel of medulloblastoma cell lines was screened for LIFRα expression by semi quantitative RT-PCR and Western blot analysis (Figure 3 4a,b). MB cell lines displayed increased expression of the LIFRα, when compared to normal brain or cerebellum (Figure 3-4b). We next sought to investigate whether LIF was co-expressed with its receptor in medulloblastoma cell lines. This analysis showed that 5 out of 6 cell lines expressed detectable levels of LIF mRNA (Figure 3-4a). In addition, three cell lines (DAOY, UW228 and PFSK) co-expressed LIF and high levels of the LIFRα (Figure 3-4a,b), indicating the presence of an autocrine loop in a subset of medulloblastoma cell lines.

The expression of the LIFRα was next determined in primary medulloblastoma, by re-analyzing DNA microarray data obtained in primary brain tumors (available from the public database Oncomine). The LIFRα was found to be over-expressed in medulloblastoma, when compared to normal cerebellum (Figure 3-4c). Together these data show that the LIFRα is over-expressed in primary medulloblastoma and medulloblastoma cell lines, and that an autocrine signalling loop involving LIF and its receptor is present in a subset of cell lines.

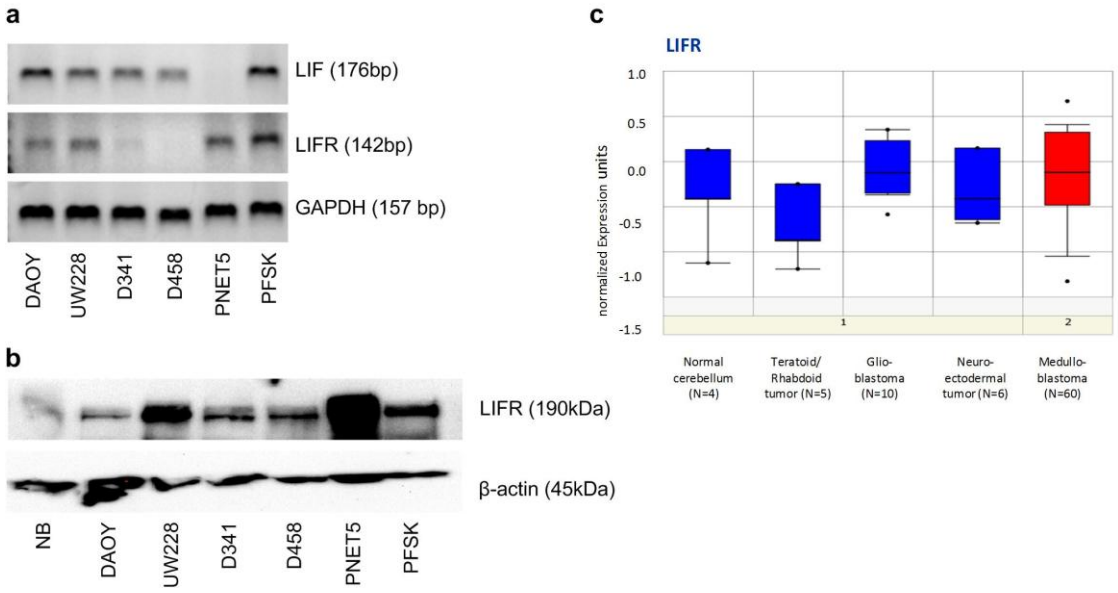

Figure 3-4 Expression of LIFRα and LIF in medulloblastoma cell lines and primary brain tumors. (a) LIF and LIFR mRNA expression in medulloblastoma cell lines was analysed by semi quantitative RT-PCR. (b) LIFRα protein levels in medulloblastoma cell lines vs. normal brain (NB) tissue were evaluated by Western blot and compared with β-actin as the loading control. (c) Box-plot illustrating the expression of LIFRα in medulloblastoma tumor samples (N = 60) versus normal cerebellum (N = 4), teratoid rhabdoid (N = 5), glioblastoma (N = 10), neuroectodermal tumor (N=6).

LIFR activation stimulates multiple intracellular signalling pathways in medulloblastoma

In order to investigate whether the LIFR is indeed functional in medulloblastoma cell lines, we analyzed the activation status of downstream signalling pathways in response to cell stimulation with oncostatin M (OSM), a potent activator of LIFR signalling. Dose-dependent activation of STAT3 could be detected, starting at a concentration of OSM of 5 ng/ml and with a maximal effect at 50 ng/ml in DAOY cells (Figure 3-5). In addition, a significant activation of Akt and Erk1/2 was observed at a concentration of 10 ng/ml and 5 ng/ml, respectively (Figure 3-5). In view of the activation of these classical downstream signalling pathways, it can be concluded that the LIFR is indeed functional in medulloblastoma cell lines.

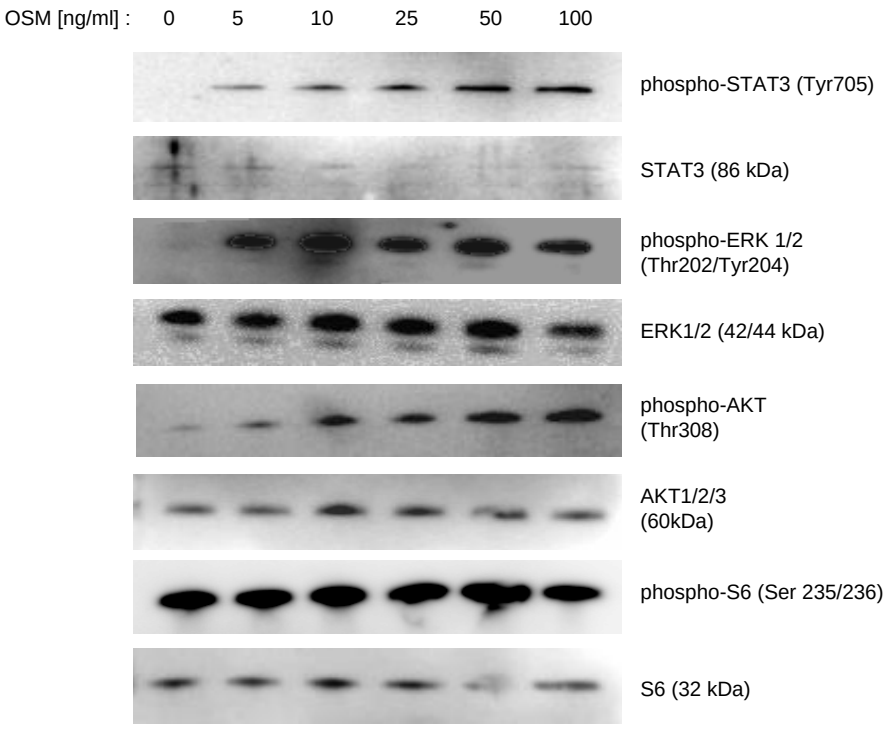

Figure 3-5 LIFR stimulation activated intracellular signalling pathways in medulloblastoma cell lines. Cell lysates of DAOY cells stimulated with oncostatin M (OSM) for 10 min were analyzed for protein expression and phosphorylation of the indicated proteins.

Impact of LIFRα down-regulation on cell proliferation and survival in medulloblastoma

We have previously shown that p110α contributes to a variety of cellular responses in medulloblastoma cell lines, including cell proliferation and survival [14]. Therefore, we studied the impact of targeting the LIFRα by RNA interference or by using a neutralizing antibody on medulloblastoma cell responses. We first used siRNA to transiently down-regulate the expression of the LIFRα in medulloblastoma cell lines (Figure 3-6a). Down-regulation of the LIFRα induced apoptosis, as shown by increased caspase-3/7 activity and led to decreased cell proliferation in DAOY and UW228 cell lines (Figure 3-6c).

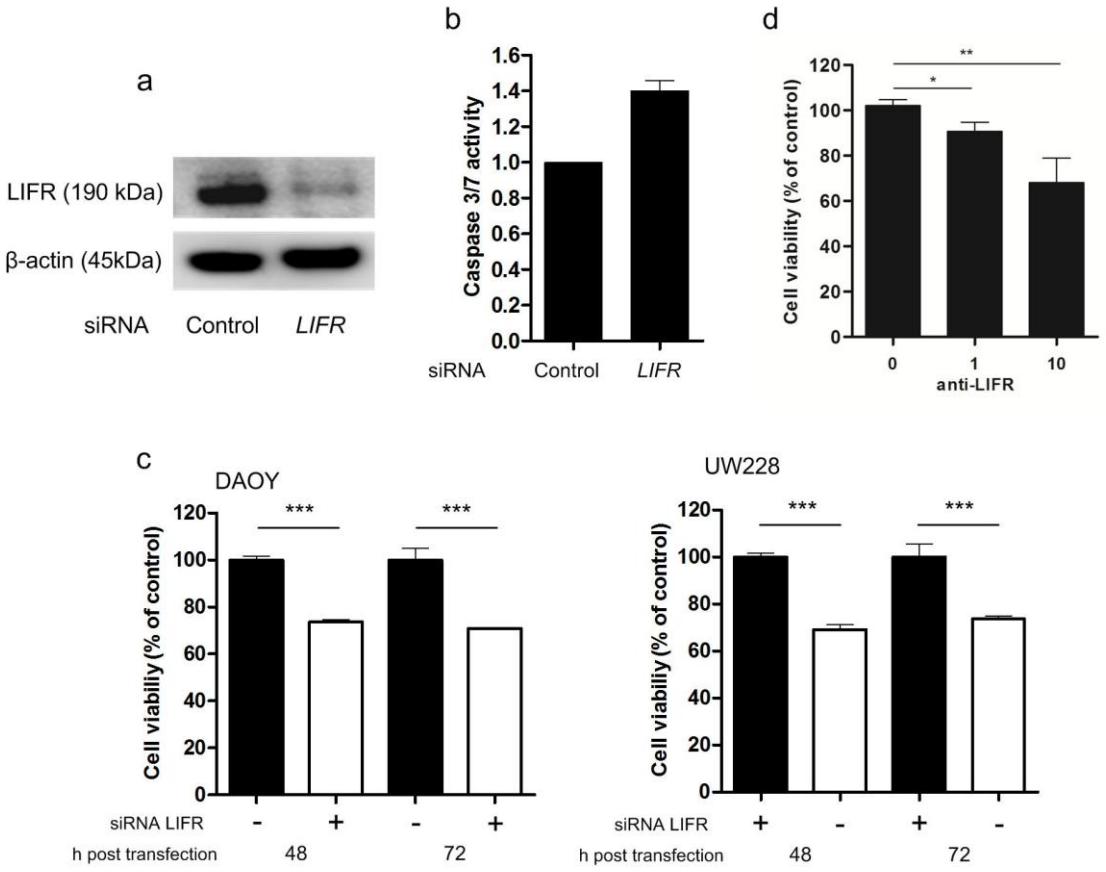

Figure 3-6 Effects of LIFRα down-regulation on medulloblastoma cell proliferation and survival. (a) Protein expression level of LIFR in DAOY cells transfected with siRNA against LIFRα compared to the control siRNA transfected cells. (b) Induction of apoptosis was evaluated in DAOY after 24h of transfection of LIFRα-siRNA or control siRNA. (c) The effects on cell viability of the siRNA-mediated target down-regulation of LIFRα (white bars) compared to control siRNA (black bars) in DAOY and UW228 cells was assessed for the indicated time points and expressed as percentages (d) Cell viability was evaluated in DAOY cells treated for 48h with an anti-LIFRα neutralizing antibody at the indicated concentrations.

These results were confirmed using a neutralizing antibody directed against the LIFRα. Antibody treatment induced a dose-dependent decrease in cell proliferation in DAOY cells (Figure 3-6d). Together these findings confirm that the LIFRα contributes to cell proliferation and protection from apoptosis in medulloblastoma cell lines.

3.5 Discussion

The PI3K/Akt pathway has been demonstrated to play a key role in medulloblastoma cell proliferation, survival, chemoresistance and migration [6, 14, 22, 23]. Mutations in *PIK3CA* were reported in primary medulloblastoma [17], as well as increased expression of *PIK3CA* at the mRNA and protein level [14]. Isoform-specific or broad specificity PI3K inhibitors have shown anti-tumor activity in medulloblastoma models in vitro and in vivo [14, 22]. In general, these agents reduced the activation status of classical PI3K downstream targets, such as Akt, mTOR, S6K or GSK-3β [24].

Targeting p110α by RNAi or isoform-specific inhibitors had more pronounced effects on medulloblastoma cell responses than in the case of p110β or p110δ, indicating a selective role for p110α in medulloblastoma [14]. This hypothesis was also confirmed by the observation that *PIK3CA* was over-expressed in primary medulloblastoma, compared to normal cerebellum, which was not the case for *PIK3CB* or *PIK3CD* [14]. In view of these observations, we hypothesized that p110α may control the expression of a selective subset of genes implicated in medulloblastoma cell proliferation and/or survival. The comparative DNA microarray analysis of medulloblastoma cell lines in which either p110α or p110δ were down-regulated by siRNA identified such a gene subset. The LIFRα was validated as a downstream target of p110α by a combination of approaches, including pharmacological inhibition or over-expression of the class IA PI3K isoform. The observation that LIFRα expression was elevated in primary medulloblastoma, in comparison to normal cerebellum further supports this model, in view of the over-expression of *PIK3CA* which we previously reported on [14].

Importantly, the LIFR was shown to be functional in medulloblastoma cell lines, since its activation stimulated classical downstream signalling pathways, such as JAK/STAT, Erk1/2 and PI3K/Akt. Moreover, the observation that medulloblastoma cell lines generally

express LIF confirms the presence of an autocrine loop in this malignant brain tumor [25, 26]. LIF is a pleiotropic cytokine, which can sustain proliferation or differentiation depending on cell type or maturation [27]. Deregulated LIF secretion has also been previously described in human cancer, such as breast cancer, rhabdomyosarcoma and medulloblastoma [25, 28, 29]. In medulloblastoma, cell lines, LIF expression was reported to be regulated by p53 [30]. Targeting LIFR downstream signalling has been evaluated in medulloblastoma. Interfering with STAT3 by using a non-peptide small molecule STAT3 inhibitor was reported to decrease cell viability and induce apoptosis in medulloblastoma [31]. A study using resveratrol also reported on the importance of STAT3 signalling in maintenance and survival of medulloblastoma cells [32].

Previous studies have reported on cross-talks between the PI3K/Akt pathway and myc family genes. GSK-3β, which is directly regulated by phosphorylation through Akt controls Myc phosphorylation and stability [33-35]. In embryonal tumors, including medulloblastoma, targeting the Akt and Erk pathways using a quassinoid analogue induced c-Myc and N-myc down-regulation [7, 8]. Inhibition of PI3K using a selective inhibitor was also reported to induce N-myc down-regulation in neuroblastoma [36]. N-myc was shown to play an important role in the regulation of PI3K-mediated VEGF secretion in neuroblastoma cells [37, 38]. Recently, myc amplification has been reported to be implicated in the resistance of tumors with activated *PIK3CA* to pharmacological inhibitors of p110α [39, 40].

Together our results delineate a novel signalling pathway from p110α to c-Myc and LIFR, which contributes to key medulloblastoma cell responses and may be further studied to develop novel targeted therapies for this common and devastating childhood malignancy.

3.6 Supplementary information

No	Network	GO Processes	Total nodes	p-Value
1	c-Myc	positive regulation of biological process, regulation of cell proliferation, regulation of developmental process, negative regulation of osteoblast differentiation, detection of mechanical stimulus involved in sensory perception of sound	25	< 0.001
2	ESR1 (nuclear)	positive regulation of estrogen receptor signalling pathway, regulation of estrogen receptor signalling pathway, positive regulation of steroid hormone receptor signalling pathway, steroid hormone receptor signalling pathway, response to unfolded protein	16	< 0.001
3	p53	negative regulation of developmental process, regulation of developmental process, regulation of multicellular organismal process, negative regulation of apoptosis, negative regulation of programmed cell death	14	< 0.001
4	STAT3	regulation of biological quality, growth hormone receptor signalling pathway, response to growth hormone stimulus, negative regulation of cellular process, response to estradiol stimulus	13	< 0.001
5	C/EBPalpha	regulation of cell proliferation, negative regulation of cell proliferation, positive regulation of cell proliferation, response to vitamin, regulation of developmental process	12	< 0.001
6	STAT1	positive regulation of developmental process , intracellular signalling cascade, regulation of cell death, regulation of programmed cell death, regulation of developmental process	11	< 0.001
7	RelA (p65 NF-kB subunit)	positive regulation of biological process, inflammatory response, positive regulation of cellular process, positive regulation of cell proliferation, regulation of cell proliferation	11	< 0.001
8	E2F1	response to extracellular stimulus, response to vitamin, negative regulation of biological process, negative regulation of cell proliferation, response to vitamin D	10	< 0.001
9	NF-kB	germinal center formation, response to wounding, inflammatory response, regulation of response to stimulus, regulation of cell proliferation	9	< 0.001
10	c-Jun	regulation of response to external stimulus, multicellular organismal process, negative regulation of cell proliferation, regulation of cell death, regulation of programmed cell death	9	< 0.001

Suppl. Table 3-1 Analysis of transcriptional networks by GeneGo Metacore. Hit list of the transcription factors whose networks of genes were mostly affected by the RNAi mediated downregulation of *PIK3CA* and *PIK3CD* in DAOY cells.

3.7 References

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CHAPTER 4

NOVEL AGENTS TARGETING THE IGF-1R/PI3K PATHWAY IMPAIR CELL PROLIFERATION AND SURVIVAL IN SUBSETS OF MEDULLO- AND NEUROBLASTOMA

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4.1 Abstract

The receptor tyrosine kinase (RTK)/phosphoinositide 3-kinase (PI3K) pathway is fundamental for cancer cell proliferation and is known to be frequently altered and activated in neoplasia, including embryonal tumors. Based on the high frequency of alterations, targeting components of the PI3K signalling pathway is considered to be a promising therapeutic approach for cancer treatment. Here, we have investigated the potential of targeting the axis of the insulin-like growth factor-1 receptor (IGF-1R) and PI3K signalling in two common cancers of childhood: neuroblastoma, the most common extra cranial tumor in children and medulloblastoma, the most frequent childhood brain tumor. By treating neuroblastoma and medulloblastoma cells with R1507, a specific humanized monoclonal antibody against the IGF-1R, we could observe cell line-specific responses and in some cases a strong decrease in cell proliferation. In contrast, targeting the PI3K p110α with the specific inhibitor PIK75 resulted in broad anti-proliferative effects in a panel of neuro- and medulloblastoma cell lines. Additionally, sensitization to commonly used chemotherapeutic agents occurred in neuroblastoma cells upon treatment with R1507 or PIK75. Furthermore, by studying the expression and phosphorylation state of IGF-1R/PI3K downstream signalling targets we found down-regulated signalling pathway activation. Together, our studies demonstrate the potential of targeting the IGF-1R/PI3K signalling axis in embryonal tumors. Hopefully, this knowledge will contribute to the development of urgently required new targeted therapies for embryonal tumors.

4.2 Introduction

Second to accidents, cancer is still the leading cause of death for children. Embryonal tumours represent approximately 30% of childhood malignancies and often display resistance to current therapeutic regimens. Therefore, embryonal tumors are associated with lower survival rates compared to other childhood cancers. Treatment failure for disseminated disease is frequent, and results in survival rates < 20%. Thus, novel therapeutic options are urgently needed for this group of tumours to improve survival rates and quality of life of patients. Embryonal tumors are dysontogenetic tumours whose pathological features resemble those of the developing organ or tissue of origin and include the entities medulloblastoma and neuroblastoma. Medulloblastoma is the most common malignant brain tumour in children and accounts for approximately 20% to 25% of all pediatric central nervous system tumours. Neuroblastoma is an embryonal tumor that originates from developing neural crest tissues. It is the most common extracanial solid tumor and is responsible for 15% of all cancer-related deaths in childhood. The fact that these cancers occur in infants and young children suggests that only a limited number of genetic changes may lead to tumor development, making these cancers an attractive model to identify new molecular targets. The development of novel targeted therapies is of particular importance for embryonal tumors, as these malignancies are orphan diseases. Common intracellular signalling pathways and chromosomal deletions including 1p36 and 11q loss have been previously identified in different embryonal tumors, including medulloblastoma and neuroblastoma [1-10].

Several intracellular signalling pathways have indeed been demonstrated to play a key role in embryonal tumor biology. Indeed, polypeptide growth factors such as insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), neuregulins and neurotrophins have been shown to control embryonal tumor proliferation, survival, differentiation and metastasis

[11-15] by binding to specific receptor tyrosine kinases (RTKs). Moreover, expression of the ErbB-2 and ErbB-4 RTKs in embryonal tumor samples was shown to correlate with reduced patient survival, while Trk receptor expression correlated with a less aggressive tumor phenotype [13]. Therefore a better understanding of the involvement of RTKs and their downstream targets in human embryonal tumor biology may yield important clues for the development of new drugs for the disease. Targeting receptor tyrosine kinases such as the IGF-1R is a promising approach to develop novel anti-cancer therapies in embryonal tumors, such as neuroblastoma and sarcoma [15-23]. Indeed the first results from clinical trials evaluating the safety and efficacy of IGF-1R neutralising antibodies in children and adolescents with embryonal tumor have been reported [24]. In this trial, the humanized IGF-1R neutralizing antibody R1507 displayed minimal toxicities and some responses in ESFT were observed [24]. Importantly, no dose-limiting toxicities were identified and the maximum tolerated dose was not reached [24]. Human embryonal tumor cells have been reported to express a variety of growth factor receptors, some of which can be activated by mutations, over-expression and/or establishment of autocrine loops [13]. Amongst these polypeptide growth factor receptors are the RTKs IGF-1R, EGFR, ALK, ErbB-2, ErbB-4, c-Kit, PDGFR, Trk and fibroblast growth factor receptor (FGFR) [25-40]. Therefore, given that embryonal tumor cells express a variety of different growth factor receptors, targeting individual receptors may not provide a successful therapeutic strategy in all embryonal tumor entities. A potentially complementary approach would be to identify signalling molecules which lies downstream of several different growth factor receptors and which are essential for transmitting their proliferative and/or survival message. Combinatorial targeting of receptor tyrosine kinases (such as the IGF-1R) and their downstream signalling mediators is a very promising approach to develop more efficient anti-cancer therapies [16, 17, 22, 41-43].

Phosphoinositide 3-kinase (PI3K) is a good example of this, playing a crucial role in controlling cell proliferation, survival and motility/metastasis downstream of many different growth factor receptors and oncogenic Ras mutants [44-47]. PI3K signalling activates a crucial intracellular signalling pathway involving phosphoinositide-dependent protein kinase-1 (PDK1), Akt, the mammalian target of rapamycin (mTOR) and the ribosomal protein S6 kinase (S6K), which controls cell growth, proliferation and survival [44-46]. The importance of PI3K/Akt/mTOR signalling in cancer is highlighted by the fact that mutations in the tumor suppressor gene PTEN occur frequently in human tumors, including glioblastoma [44, 48-50]. PTEN is a phosphatase that antagonizes the action of PI3K by de-phosphorylating the D-3 position of polyphosphoinositides [44, 48, 49]. Reduced expression of PTEN resulting in activation of PI3K signalling was recently described in embryonal tumors such as medulloblastoma and neuroblastoma [51, 52]. Moreover, various reports have described activating mutations in the PIK3CA gene encoding the catalytic p110α isoform of PI3K in a variety of human cancers, including, breast, colon and ovarian cancer, as well as embryonal tumors [50, 53, 54]. In addition, PI3K/Akt/mTOR signalling has been demonstrated to mediate the proliferation of embryonal tumor cells [55, 56] and to contribute to signalling by ErbB-2 and IGF-1R [57-59]. Activation of Akt was also reported in embryonal tumors, correlating with poor outcome in some entities [60]. Thus, targeting the PI3K/Akt/mTOR signalling pathway may represent an attractive novel approach to develop novel therapies for embryonal tumors [61]. Indeed, there now exist multiple pharmacological inhibitors of the PI3K/Akt/mTOR pathway which have entered clinical trials for adult and pediatric cancer [43, 45, 47, 62-64]. The PI3K/Akt/mTOR pathway is also an important contributor to the resistance of human tumors to drugs targeting receptor tyrosine kinases [65-67]. Inhibitors of the PI3K/Akt/mTOR signalling pathway have also been shown to be effective in combination with IGF-1R inhibitors [20, 42].

In the present report, we have evaluated the anti-proliferative potential of the humanized anti-IGF-1R antibody R1507 and of PIK75, a class IA PI3K inhibitor, in medulloblastoma and neuroblastoma cell lines. We present evidence, that these agents are effective as monotherapies in subsets of embryonal tumor cell lines and can be effectively combined with standard chemotherapeutic drugs.

4.3 Materials and methods

Cell Culture

Human neuroblastoma cell lines were kindly provided by Dr Brodeur, Children's Hospital of Philadelphia. The cells were grown in RPMI (Life Technologies/Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin/L-glutamine and passaged every 3-5 days by trypsinization.

The medulloblastoma cell lines' provenience has been previously described [68]. DAOY, UW-228 and PFSK human cell lines were purchased from the American Type Culture Collection. D341 Med and D458 Med medulloblastoma cells were the kind gift of Dr. Henry Friedman (Duke University, Durham, NC). Cell lines that were not purchased from the American Type Culture Collection in 2009 were tested for their authentication by karyotypic analysis using molecular cytogenetic techniques, such as comparative genomic hybridization. DAOY medulloblastoma cell line was grown in Richter's MEM Zinc option medium (Invitrogen) with 10% FCS (fetal bovine serum; Sigma) and penicillin/streptomycin (Invitrogen). PFSK primitive neuroectodermal tumor (PNET) cell line was grown in RPMI 1640 (Invitrogen) with 10% FCS and penicillin/streptomycin/L-glutamine. The UW-228 medulloblastoma cell line was grown in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) with 10% FCS and penicillin/streptomycin/L-glutamine. D341 Med and D458 Med medulloblastoma cell lines were grown like DAOY but with the addition of 100M non-essential amino acids (GIPCOTM MEM Invitrogen). All cells were grown in a humidified atmosphere at 37° and 5% CO2.

Reagents

The PI3K inhibitors PIK75 was dissolved in DMSO (Sigma, Buchs, Switzerland) at 10 mM and diluted into cell culture medium just before use. R1507, a fully human IgG1 monoclonal antibody to IGF-1R, was

obtained from La-Roche, and was diluted directly into the medium immediately before use. The chemotherapeutic agents cisplatin (Bristol-Myers Squibb), doxorubicin (Pfizer) and etoposide (Calbiochem) were used in combination with PIK75 or R1507 at the indicated concentrations.

Cell Proliferation

Neuroblastoma and medulloblastoma cells were seeded in 96-well plates at a density of 3’000-10´000 cells/well and grown for 48-118 hrs in cell culture containing high (10%) serum. Cell proliferation was analyzed by the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s instructions.

Western blotting

Cell lysates were prepared in RIPA buffer (50mM Tris-Cl, pH 6.8, 100mM NaCl, 1% Triton X-100, 0.1% SDS) supplemented with Complete Mini Protease Inhibitor Cocktail (Roche Applied Sciences) and with the phosphatase inhibitors β-glycerophosphate (20mM) and Na3VO4 (200 mM) and normalized using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences, GE Healthcare, UK) and immunoblotted with the indicated antibodies prior to chemiluminescent detection (SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA). Antibodies specific for IGF1Rβ, PI3K p110α, Akt1/2/3, ERK 1/2, S6 protein (Santa Cruz Biotechnology, CA, USA), phospho-ERK1/2 (Thr202/Tyr204), phospho-Akt (Ser473; Thr308), phospho-S6 (Ser235/Ser236; Ser240/Ser244).

4.4 Results

Anti-proliferative activity of R1507 and PIK75 in panels of neuroblastoma and medulloblastoma cell lines

We have previously described panels of neuroblastoma and medulloblastoma cell lines, which were characterized for expression of components of the IGF-1R/PI3K signalling pathway [20, 69, 70]. In the present study, the impact of the humanized anti-IGF-1R antibody R1507 was evaluated on cell proliferation in vitro in the panels of neuroblastoma and medulloblastoma cell lines (Figure 4-1). The antibody displayed anti-proliferative activity in 2 out of 8 neuroblastoma cell lines, namely SH-SY5Y and LAN1 (Figure 4-1a and Figure 4-6a). In SH-SY5Y, R1507 induced a maximal decrease in cell proliferation of 70% at 12.5μg/ml (Figure 4-1a). In LAN1 a maximal activity of 30% reduction in cell proliferation was observed (Figure 4-6a). R1507 showed anti-proliferative activity in 2 out of 5 medulloblastoma cell lines, namely PFSK and D458 (Figure 4-1b). In D458, a maximal decrease in cell proliferation (60%) was observed at 15μg/ml, while in PFSK the maximal effect was 40% inhibition of the response (Figure 4-1b). In NB and MB cell lines, the activity of R1507 was cell-line specific and the antibody had a profile similar to the IGF-1R tyrosine kinase inhibitor NVP-AEW541 [20]. NVP-AEW541 was more active in SH-SY5Y and LAN-1 than in other neuroblastoma cell lines [20], and PFSK were more sensitive to NVP-AEW541 than DAOY and UW228 cells.

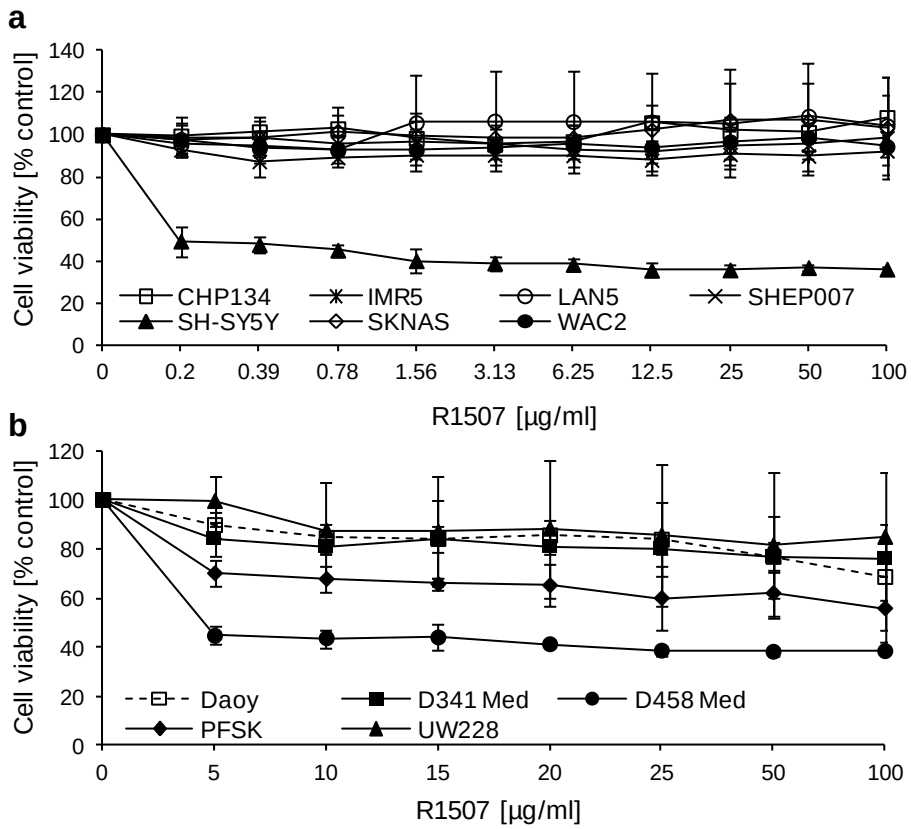


Figure 4-1 The effect of R1507 on (a) NB and (b) MB cell lines after 48 hours. Error bars represent ±S.D. of means from 3 experiments, each with 6-8 replicates. NB different scales.

The impact of the class IA PI3K inhibitor PIK75 was evaluated on cell proliferation in vitro in the panels of neuroblastoma and medulloblastoma cell lines. The inhibitor displayed anti-proliferative activity in the neuroblastoma cell line panel, with IC_{50} values in the range of 50-100 nM (Figure 4-2a). In medulloblastoma cell lines, PIK75 was more active in D341 and D458 cells (IC_{50}= 20 nM) than in UW228 cells. The activity of PIK75 in DAOY and PFSK cells was previously described in [69].

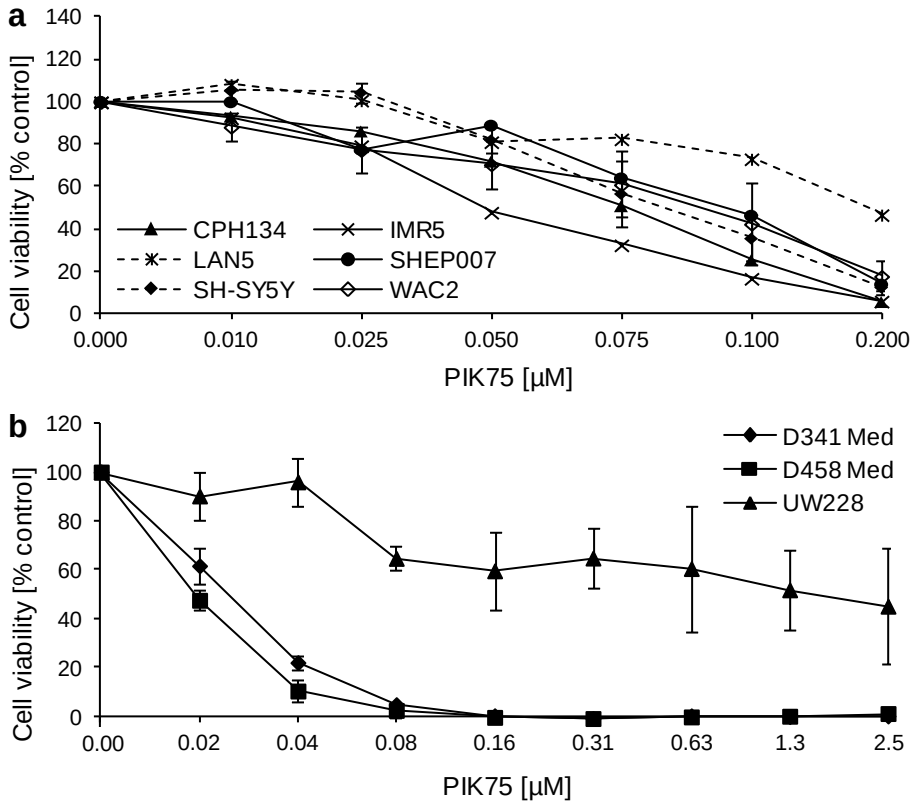


Figure 4-2 The effect of PIK75 on NB and MB cell lines. a) neuroblastoma cells after 48 hours. b) D341 and D458 Med cells after 118 hours, UW228 cells after 70 hours. Error bars represent ±S.D. of means from 1 to 3 experiments, each with 4-8 replicates. NB different scales.

Impact of R1507 and PIK75 on intracellular signalling pathway activation

The impact of R1507 and PIK75 on the activation status of the Akt/mTOR pathway in NB cell lines was investigated by Western blot analysis (Figure 4-3). R1507 strongly affected the activation status of Akt and the phosphorylation of the mTOR downstream target ribosomal S6 protein in both NB and MB cell lines. (SH-SY5Y in Figure 4-3a, LAN1 cells Figure 4-3b as well as in medulloblastoma PFSK cells Figure 4-3c). Concentrations of 6.25-100 µg/ml R1507 all reduced Akt Ser 473 phosphorylation, whereas only concentrations of more than 6.25 or 12.5 were needed to reduce S6Ser235/236 phosphorylation.

Also PIK75 was able to inhibit Akt/mTOR activation, as seen on the decreases in the phosphorylation of S6 protein in both NB cell lines treated, although only in LAN-1 cells the phosphorylation of AktSer473 was affected (Figure 4-3b).

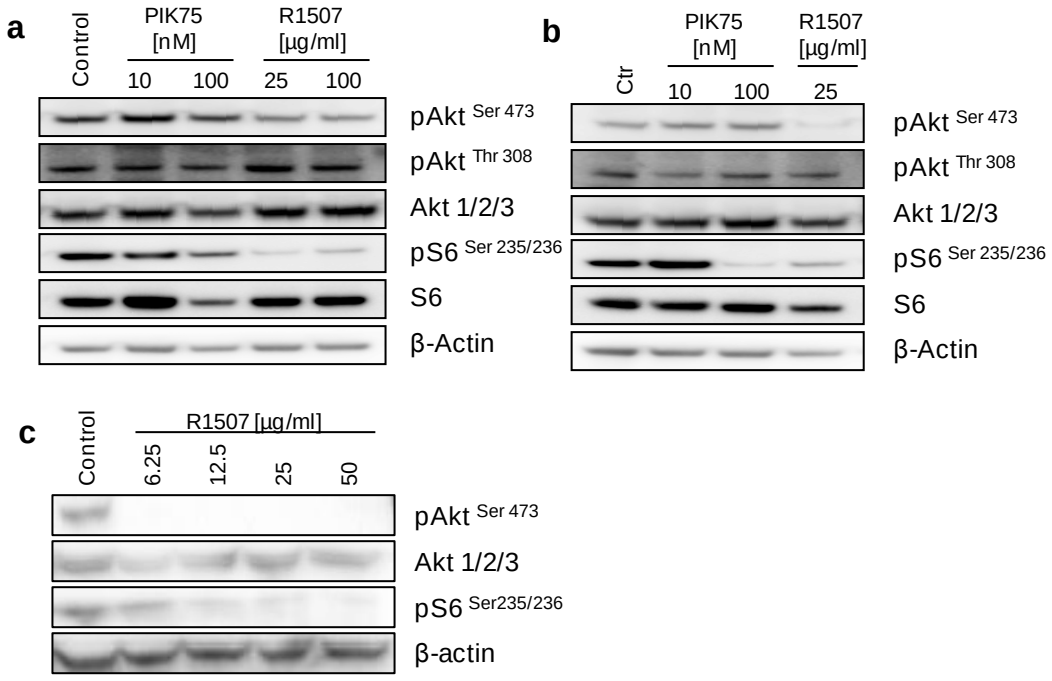


Figure 4-3 Western blots showing change in phosphorylation in response to different treatments. The effect of treatment with PIK75 and R1507 on phosphorylation of Akt and S6 downstream in the PI3K/Akt/mTOR signalling pathway in neuroblastoma (a) SH-SY5Y, (b) LAN1 and (c) PFSK cells.

The inhibitory effects of PIK75 in Akt/mTOR signalling in MB cell lines cells were previously described in [69] and correlate with the effects observed in neuroblastoma cells.

Combination of R1507 with standard chemotherapeutic agents in neuroblastoma and medulloblastoma cell lines

We have previously shown that the IGF-1R tyrosine kinase inhibitor NVP-AEW541 enhances the effects of cisplatin on cell proliferation and apoptosis in neuroblastoma cell lines [20]. In support of this

finding, the concomitant treatment of the R1507-responsive SH-SY5Y neuroblastoma cell line with R1507 and cisplatin resulted in additive effects on cell proliferation (Figure 4-4a). For neuroblastoma WAC2 cells that did not respond to R1507 in single treatment (Figure 4-1), there was no additional effect of R1507 in combination with cisplatin, doxorubicin or etoposide (Figure 4-1a). In medulloblastoma PFSK and UW228 cells, the combination of R1507 and cisplatin was more effective than the single agents (Figure 4-4b). This is not surprising for PFSK, that also showed sensitivity to R1507 alone, but in UW228, cisplatin seems to confer R1507 sensibility. In R1507-insensitive DAOY cells no such effect was observed (Supplementary Figure 4-1).

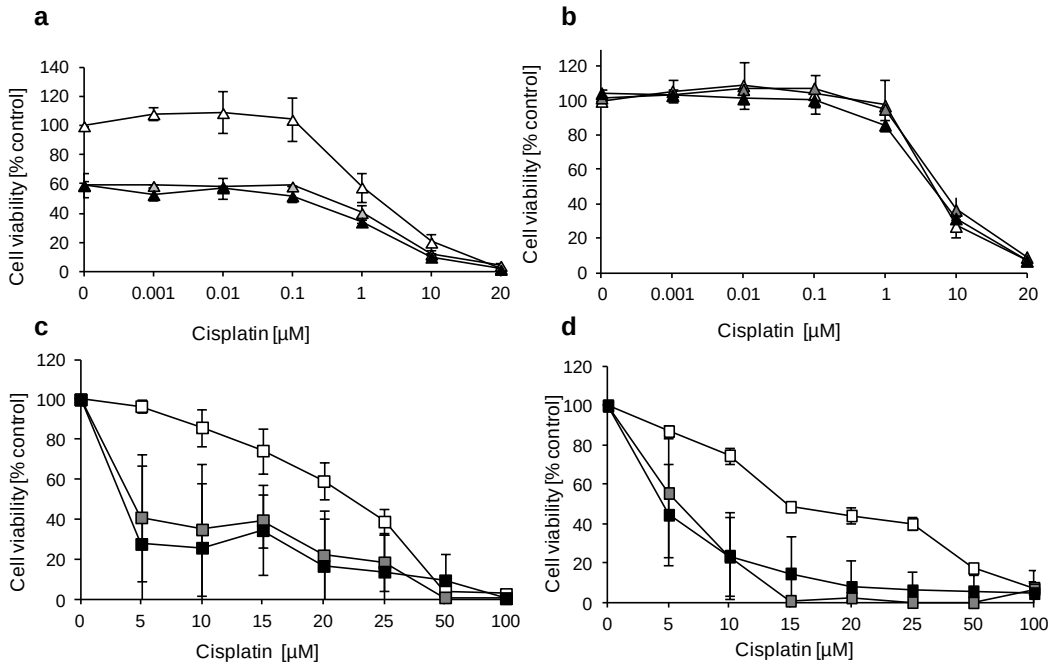


Figure 4-4 Combinational treatments of R1507 with chemotherapy in NB and MB cell lines after 48 hours. a) SH-SY5Y and b) WAC2 c) PFSK cells and d) UW228 cells treated with R1507 (white squares 0 µg/ml, grey squares 25 µg/ml and black squares 50 µg/ml) and cisplatin. Error bars represent ±S.D. of means from 1 to 3 experiments, each with at least 4 replicates. For combination treatments of WAC2 and DAOY cells which are irresponsive to R1507, see Supplementary Figure 4-1.

Combination of PIK75 with standard chemotherapeutic agents in neuroblastoma cells

In the neuroblastoma cell lines LAN1 and WAC2, the concomitant treatment with PIK75 and doxorubicin or etoposide resulted in additive effects on cell proliferation (Figure 4-5). At selected, relatively low concentrations, where PIK75, doxorubicin or etoposide alone reduced cell proliferation to 60-80%, combination treatments of PIK75 and one of the chemotherapeutic agents brought reductions to 40-50%. Our previous work on medulloblastoma has shown that PIK75 sensitizes medulloblastoma cell lines to doxorubicin [69].

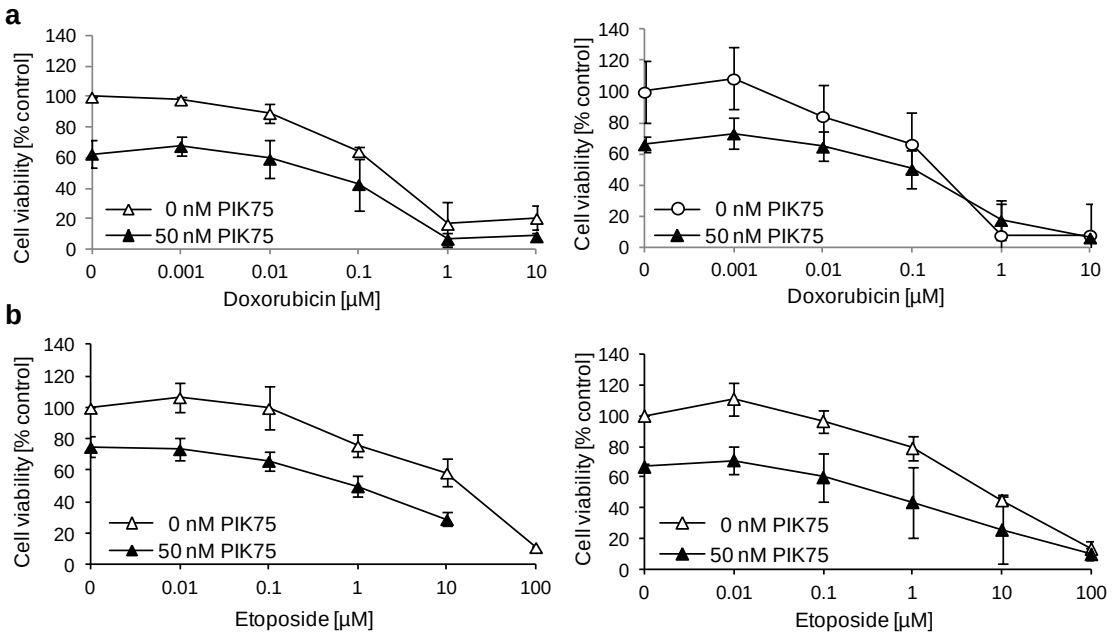


Figure 4-5 Additive effects of treatment with PIK75 in combination with different chemotherapies in NB cell lines after 48 hours. a) Combinatory treatment of PIK75 with doxorubicin in LAN-1 (right) or WAC-2 (left) cells. b) Combinatory treatment of PIK75 with etoposide in LAN-1 (right) or WAC-2 (left) cells. Error bars represent ±S.D. of means from 3 experiments, each with at least 4 replicates.

Activity of R1507 and PIK75 in chemoresistant NB cell lines

We next investigated whether R1507 or PIK75 had also antiproliferative effects in neuroblastoma cell lines with acquired resistance to standard chemotherapeutic agents. R1507 displayed no significant anti-proliferative activity in LAN-1 cells with acquired resistance to doxorubicin (Figure 4-6a). In contrast, PIK75 displayed almost comparable anti-proliferative activity in either parental LAN-1 or their chemoresistant counterparts (Figure 4-6b). Western blot analysis of the protein expression of LAN1R cells showed that these cells express reduced levels of IGF-1R compared to the parental cell line LAN1. In addition, the phosphorylation levels of ERK1/2 and AKT at the positions Ser 473 and Thr308 were also lower in LAN1R than in LAN1 (Figure 4-6c).

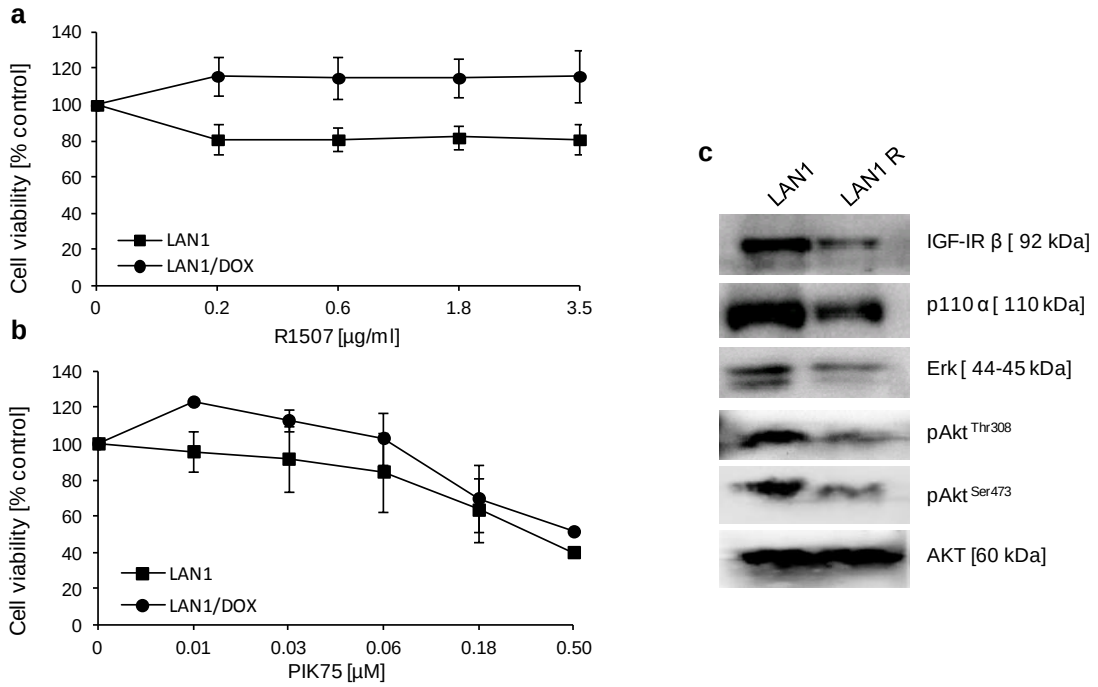


Figure 4-6 Sensitivity to R1507 and PIK75, and presence of IGFR in neuroblastoma cell lines LAN1 and LAN1R, a LAN1 cell line resistant to doxorubicin. a) R1507 treatment for 48 hours. b) PIK75 treatment for 48 hours. Error bars represent ±S.D. of means from 3 experiments, each with 3 replicates, except that there was only one experiment with 500nM PIK75 in b. c) Western blot analysis of the indicated proteins in LAN1 and LAN1R cell extracts.

4.5 Discussion

In the present report we have evaluated the anti-proliferative activity of the humanized anti-IGF-1R antibody R1507 in the embryonal tumors neuroblastoma and medulloblastoma cell lines in vitro. As a single agent, R1507 was effective in a subset of neuro- and medulloblastoma cell lines, while a majority of cell lines did not respond. The profile of R1507 in neuro- and medulloblastoma was similar to NVP-AEW541 in terms of the identity of the cell lines which were sensitive to the single agent [20]. In neuroblastoma cell lines that were sensitive to R1507 as single agent, the effects of R1507 and chemotherapy (cisplatin, doxorubicin and etoposide) were additive, a result which was also observed with NVP-AEW541 [20]. However, neuroblastoma cells which were not sensitive to R1507, showed also no additive effects in cell growth inhibition to when combined with chemotherapies. By contrast, in medulloblastoma R1507 showed strong synergistic effects with cisplatin not only in MB cells which were initially sensitive to R1570 (PFSK), but also MB cells which were insensitive to R1507 as a single agent (UW228). Analysis of the mechanisms of action revealed that R1507 inhibits cell growth by attenuation of the AKT/mTOR signalling pathway in neuroblastoma and medulloblastoma cells. Similar observations were obtained by inhibition of IGF1R with NVP-AEW541 [20].

Our previous work using RNAi targeting of class IA PI3K isoforms has revealed that targeting these enzymes in neuroblastoma and medulloblastoma cell lines can induce apoptosis and decrease cell proliferation [69,70]. These results are supported by the findings presented here, which show that PIK75 displays a broad anti-proliferative activity in neuroblastoma cell lines. Also in medulloblastoma, we observed that PIK75 has anti-proliferative activity, but one cell line (UW228) was rather resistant to the drug. The exact mechanism(s) underlying this observation are at present unclear, but may be caused by an enhanced activation of Erk1/2. A decrease in activity of class IA PI3K inhibitors has been observed previously in

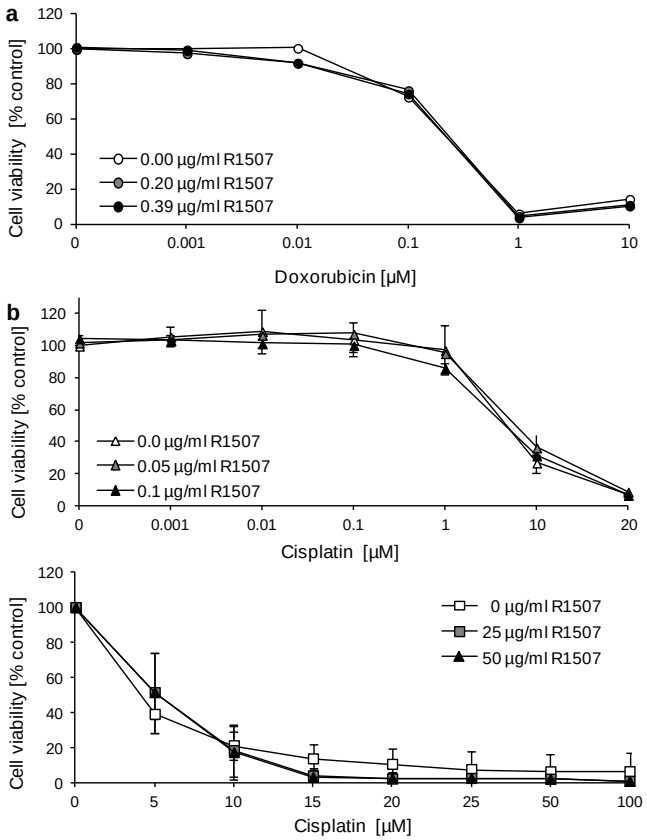
cell lines with mutant KRAS and attributed to the enhanced activation of the Erk pathway [71]. The combination of PIK75 with chemotherapy (doxorubicin and etoposide) showed enhanced cell growth inhibition as compared with single agent treatment in neuroblastoma cell lines. Consistent with these findings, a recent report demonstrated that PI103 a dual inhibitor against p110α and mTOR strongly synergizes with various chemotherapeutics including doxorubicin, etoposide and cisplatin [72]. In medulloblastoma, our previous work has also demonstrated improved the anti-proliferative effects for PIK75 in combination with different chemotherapeutic agents [69].

Because neuroblastoma and medulloblastoma cells may express a variety of different growth factor receptors, we and others have postulated that targeting individual receptors may not always provide the best therapeutic option [20,70]. To overcome this problem, an alternative approach was proposed, which is based on targeting downstream signalling molecules that are regulated by different growth factor receptors to transmit the proliferative message. Our findings support this approach, since we observed that generally a bigger number of NB and MB cell lines most likely responded to PIK75 than to R1507. Importantly, PIK75 effectively inhibited proliferation in a chemoresistant neuroblastoma cell line in a comparable manner as in the parental cell line, demonstrating its broad anti-proliferative activity. By contrast, R1507 was ineffective in the chemoresistant neuroblastoma cells, which was most likely caused by loss of expression of the IGF-1R. The AKT/mTOR pathway was also found to be deregulated in the chemoresistant cells, pointing that this signalling pathway may not be essential for the acquired chemoresistant phenotype of the cells. However, our previous findings in medulloblastoma cells showed elevated levels of phosphorylated AKT as a consequence of short time exposure with doxorubicin [69]. The molecular mechanisms underlying these observations are at present unclear, but may be of importance, in view of the fact that some

clinical trials have been initiated with R1507 in patients previously treated with chemotherapy.

The evaluation of the anti-proliferative potential of the humanized anti-IGF-1R antibody R1507 and the class IA PI3K inhibitor PIK75 presented here provides important insights that may be considered for the design of further clinical-oriented studies aimed at targeting IGF-1R/PI3K in embryonal tumors.

4.6 Supplementary Information



Suppl. Figure 4-1 Effects of the treatment of R1507 in combination with chemotherapy in NB and MB cell lines after 48 hours. a) Combination with doxorubicin in WAC2 cells b) Combination with cisplatin in WAC2 and DAOY cells. Error bars represent ±S.D. of means from 1 to 3 experiments, each with at least 4 replicates.

4.7 References

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CHAPTER 5

CONCLUSIONS AND OUTLOOK

The aim of this thesis was the identification and characterization of novel genes, which are responsible for promoting cell survival and chemoresistance in NB and MB, and which could represent potential therapeutic targets for future patient treatments. The motivation for this study was the poor survival rates in patients with high risk features. Many efforts have been done in the past decades to better characterize the biology of these tumors. In particular, large-scale approaches, such as DNA microarrays, have been carried out aimed at identifying specific tumor finger prints [1-3]. An amount of data was promptly generated, indicating the differential expression or mutational status of several genes in cancer cells compared to normal cells. But in most of the cases it remained to be discovered, which was the particular function of the identified genes. It became more important to design new, or adapt the existing large-scale approaches to address the functionality of these deregulated genes in cancer cells.

At the time this project was initiated, there were no functional high-throughput screens that interrogated oncogenic phenotypes in neuroblastoma cells reported in the literature. Contemporary to this study, other functional RNAi screens have been performed [4, 5], using shRNA or siRNA libraries targeting different subsets of genes to study

loss-of-function responses. Recently, an RNAi screen seeking for kinases that are essential for neuroblastoma survival was reported [4]. However, a chemosensitizing RNAi screen like ours (described in chapter 2), designed to identify kinases that modulate response to chemotherapies commonly used in the clinics has not been reported so far in neuroblastoma. Since chemotherapy is still the first-line treatment in the clinics and chemoresistance is a major cause of treatment failure and poor outcomes [6], this study is of great importance for the development of novel targets for combinational approaches.

The kinome-wide RNAi screen presented in this thesis identified novel potential drug targets involved in neuroblastoma survival and resistance to cisplatin. Among the survival kinases, the different isoforms of the Protein kinase C family PKC-β1, PKC-δ and PKC-ζ, were shown to play an important role in neuroblastoma survival. The previously reported survival RNAi screen in NB identified and validated CHK1 as a novel survival kinase [4]. In our cell lines, CHK1 does not seem to play a main role in cell survival, which might be explained due to the biological variability within the cell lines used in both screens. Nevertheless, the kinases BMPR1A and RPS6KB1 were commonly identified as survival kinases in both studies, suggesting that they have a more general role in neuroblastoma biology, and indicating that further studies would be interesting. Among the chemosensitizer kinases, the fibroblast growth factor receptor 2 (FGFR2) has emerged as the most promising candidate and was further validated.

Although RNAi screenings have shown to be valuable tools for the low-cost and -time consuming identification of genes associated with specific phenotypes, this technique is only feasible in *in vitro* models. The problematic of translating the results to a clinical setting remains challenging. To address this issue, we have accessed a data base of DNA microarrays comprising tumor samples of 251 NB patients. The expression of *FGFR2* was shown to correlate with advanced tumor stage. In addition, IHC analysis of NB primary samples demonstrated

also the expression of FGFR-2, as well as the ligand FGF-2, at a protein level. These findings suggest that targeting FGFR-2 in neuroblastoma might have a clinical relevance.

Consistent with our study, a previous study detected FGF-2 over-expression in neuroblatoma tumors at advanced stages (3 and 4) compared with low-stage tumors (stages 1, 2, and 4S) [7], but the mechanistic insights or the therapeutic potential of targeting FGF-2/FGFR signalling in neuroblastoma were not yet elucidated. In the present study, we hypothesize that FGFR2 is activated by an autocrine loop involving FGF-2 in NB cells. We demonstrated that primary NB and NB cell lines are able to synthesize FGF-2. Stimulation of NB cells with FGF-2 resulted in FGFR2 activation, which finally led to the up-regulation of the anti-apoptotic factor BCL2. PKC isoforms were shown to play a major role in FGFR2 signalling to BCL2, in contrast to other signalling pathways, such as PI3K/mTOR and ERK. Our studies performed in NB cell lines with acquired chemoresistance demonstrated that FGF-2, FGFR2 and BCL2 are up-regulated, highlighting the importance of this factors for rendering the cells resistance against chemotherapies.

Overexpression of BCL2 represents one of the mechanisms that contribute to cisplatin resistance [8]. It is therefore not surprising that the down-regulation of BCL2, mediated by FGFR2 inhibition, sensitizes cells to cisplatin induced cell death. The analysis of the mechanisms of action downstream of FGFR2 was also of importance, because it led to the identification of other possible targeting sites on the signalling pathway that might have a pharmacological potential, as well. Beside the inhibition of FGFR2 with SU-5402, also inhibiting the downstream effectors using the PKC inhibitors Rottlerin and Enzastaurin showed anti-proliferative effects in neuroblatoma. Furthermore, the BCL2 inhibitor ABT-737 has shown to have a great impact in growth inhibition in NB cells with acquired chemoresistance. This observation is in agreement of a previous study, in which has also

documented the potential of targeting BCL2 family proteins with ABT-737 in NB [9-11].

As described in the introduction, genetic alterations in FGFR2 have been implicated in various cancer types, resulting in aberrant FGFR2 signalling activation, due to the release of FGFR2 from auto inhibition, or through creation of FGF signalling autocrine loops [12]. Several pharmacological inhibitors against FGFR are currently being tested in clinical trials, including ZD-4547, BGJ398 and FP-1039, which are entering Phase I or II for the treatment breast, gastric or endometrial cancers, or advanced solid cancers [13]. Within this thesis, we have only analyzed the pharmacological potential of the FGFR2 inhibitor SU-5402 in neuroblastoma. This inhibitor has also affinity for FGFR1 and FGFR3. Because of the homology among the isoforms, it is difficult to design isoform-specific small molecule inhibitors. Most of the available FGFR inhibitors target more than one isoform. Our studies with siRNAs point that the down-regulation of *FGFR2* is sufficient to sensitize NB cells to cisplatin treatment. However, it remains to be studied, whether the inhibition of FGFR1 or FGFR3 by SU-5402 also contribute to neuroblastoma chemosensitization. In terms of future perspectives, other inhibitors against FGFR could be also tested in neuroblastoma. Of particular interest are those which are being tested in clinical trials for other cancer types. Further, it would be interesting to see whether the *in vitro* anti-proliferative effects of SU-5402 or maybe other FGFR2 inhibitors can be reproduced in *in vivo* models. In this context, also the inhibitors enzastaurin and ABT-737 represent attractive targets for further *in vivo* studies.

As extensively described in the introduction and reviewed in the appendix, the PI3K signalling pathway plays a major role in neuroblastoma and medulloblastoma biology. Therefore, the goal of the next projects (described in chapter 3 and 4) was to investigate the potential of targeting IGF-1R and PI3K in neuroblastoma and medulloblastoma. By treating NB and MB cell lines with R1507, a specific humanized monoclonal antibody against the IGF-1R and

PIK75 a small molecule inhibitor against PI3K p110α, we could observe cell line-specific responses, for some cell lines a strong decrease in cell proliferation. Additionally, sensitization to commonly used chemotherapeutic agents occurred in neuroblastoma cells upon treatment with R1507 or PIK75. The findings of this study, together with a number of previous studies that investigated the potential of targeting the PI3K signalling pathway in other cancers *in vitro* and *in vivo*, lead us to the conclusion that PI3K inhibitors may have an effect in tumor growth inhibition, but used as single agents, the effects are too modest, particularly to be adopted as a standard regime for patient treatment [14]. However, their application in combination with other chemotherapies have yielded so far better results and might be beneficial when applied in patients [14].

The more we know about the mechanisms underlying PI3K signalling, the better we will be able to predict which the best combinations to optimize treatment response are. To gain a better knowledge of PI3K downstream effector genes, we analyzed the gene expression profiles of MB cells upon RNAi mediated down-regulation of *PIK3CA* or *PIK3CD*, encoding for the PI3K catalytic isoforms p110α and p110δ. By using bioinformatic tools, a comparative analysis of the sets of genes, which were differentially expressed, revealed a transcriptional network of genes involving c-Myc. C-Myc is a well characterized oncogene and a marker of poor prognosis in medulloblastoma [15]. There are some reports suggesting crosstalks between PI3K/AKT signalling and c-Myc [16-18]. In large-cell MB, PI3K/GSK3β were shown to be involved in HGF-induction of c-Myc. Recently, *MYC* amplification was associated with resistance to pharmacological inhibitors of p110α [19].

Being a transcription factor, direct pharmacological targeting of c-Myc has proven difficult [20]. Therefore, targeting c-Myc responsive genes represents an option. The transcriptional network of c-Myc included LIFR as target gene, which was one of the top deregulated genes upon *PIK3CA* down-regulation. Different lines of evidence validated LIFR

as a p110α downstream target. LIFR was on one hand down-regulated upon pharmacological inhibition or RNAi mediated down-regulation of p110α and on the other hand up-regulated upon p110α up-regulation. A c-Myc inducible cell line model confirmed that LIFR transcription is mediated by c-Myc. Although the analysis of the DNA sequence of LIFR showed promoter binding sites for c-Myc, this remains to be confirmed experimentally, for example by ChIP assay, whether c-Myc directly binds to LIFR.

There are some reports about the role of LIFR in human cancers, including glioblastoma and medulloblastoma, although its role has not been extensively studied [21, 22]. A possible role for LIFR in medulloblastoma was suggested by the elevated expression of LIFR in primary MB and MB cell lines as compared with normal cerebellum. Activation of LIFR by LIF or OSM is known to activate classical signalling cascades such JAK/STAT, MAPK and PI3K/AKT which can influence cell survival, proliferation and differentiation. Consistent with our data, a previous study reported deregulated LIF expression in MB [22]. In terms of mechanisms, our study showed for the first time a functional role for LIFR in medulloblastoma. The therapeutic potential of targeting LIFR in medulloblastoma is underlined by the fact that its down-regulation by siRNA impaired cell growth in a similar magnitude as *PIK3CA* down-regulation. Confirming these observations, a monoclonal antibody against LIFR-α has demonstrated to have inhibitory effects on medulloblastoma survival and proliferation.

Being a downstream effector of the famous oncogenes and outcome predictors p110α and c-Myc in medulloblastoma, LIFR definitely represents an interesting target for further analysis. In the first instance, more MB cell lines could be tested, in order to identify a subgroup of responding MB cells. Also the combination with chemotherapies or PI3K inhibitors could be analyzed for synergistic effects. If successful, these studies could initiate the development of novel strategies to target LIFR *in vivo* and hopefully later also applied in clinical settings.

The use of large-scale approaches in NB and MB allowed us to the identification of novel target genes. In neuroblastoma, the potential of combining FGFR2 inhibition with chemotherapy was presented. In medulloblastoma, LIFR was identified as an important regulator of cell survival downstream of p110α/c-Myc. Further studies focused on the therapeutic potential of FGFR2 and LIFR would be definitely of great interest. Nevertheless, only FGFR2 and LIFR have been validated from our screens. The high throughput techniques used in this work allowed the generation of an amount of data, from which other interesting candidate genes could be validated. Such studies would also contribute to our knowledge of the molecular mechanisms undergoing in MB and NB. Building a solid knowledge about the complexity of the signalling networks in cancer cells will hopefully one day provide us the appropriate tools to combat this devastating disease.

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RNAi screen identifies new chemosensitizer kinases in Neuroblastoma, Fabiana Salm, Anna L. Buccarello, Anubrata Ghosal, Alexandre Arcaro. Awarded Poster.

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APPENDIX (REVIEW ARTICLE)

THE PHOSPHOINOSITIDE 3-KINASE SIGNALLING PATHWAY AS A THERAPEUTIC TARGET IN GRADE IV BRAIN TUMORS

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Review Manuscript

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Abstract

Brain tumors comprise a wide variety of neoplasia classified according to their cellular origin and their morphological and histological characteristics. The transformed phenotype of brain tumor cells has been extensively studied in the past years, achieving a significant progress in our understanding of the molecular pathways leading to tumorigenesis. It has been reported that the phosphoinositide 3-kinase (PI3K)/AKT signalling pathway is frequently altered in grade IV brain tumors resulting in uncontrolled cell growth, survival, proliferation, angiogenesis and migration. This aberrant activation can be explained by oncogenic mutations in key components of the pathway or through abnormalities in its regulation. These alterations include overexpression and mutations of receptor tyrosine kinases (RTKs), mutations and deletions of the phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*) tumor suppressor gene, encoding a lipid kinase that directly antagonized PI3K activity, and alterations in Ras signalling. Due to promising results of preclinical studies investigating the PI3K/AKT pathway in grade IV brain tumors like glioblastoma and medulloblastoma, the components of this pathway have emerged as promising therapeutic targets to treat these malignant brain tumors. Although an arsenal of small molecule inhibitors that target specific components of this signalling pathway are being developed, its successful application in the clinics remains a challenge. In this article we will review the molecular basis of the PI3K/AKT signalling pathway in malignant brain tumors, mainly focusing on glioblastoma and medulloblastoma, and we will further discuss the current status and potential of molecular targeted therapies.

Keywords:

AKT, clinical trial, glioblastoma, medulloblastoma, mTOR, phosphoinositide 3-kinase, PTEN

INTRODUCTION

Grade IV Brain Tumors – Glioblastoma and Medulloblastoma

Malignant brain tumors represent a large diversity of neoplasms with an incidence of 7.2 cases per 100’000 persons per year in the United States (between the years 2004 and 2006) [1]. These tumors can be classified according to their cellular origin, histology and morphology. According to the 2007 World Health Organization classification, the main classes of tumors of the central nervous system are tumors of neuroepithelial tissue, tumors of cranial and paraspinal nerves, tumors of the meninges, lymphomas and haematopoietic neoplasms, tumors of the sellar region and metastatic tumors [2]. Among others, the group of neuroepithelial tissue tumors includes astrocytic tumors like glioblastoma, the most common malignant brain tumor in adults (age 45-84 years), and embryonal tumors like medulloblastoma, the most common malignant brain tumor in early childhood (age 0-4 years) [1-2].

The broad category of glioma includes astrocytoma, glioblastoma, oligodendroglioma and others, and accounts for 32% of all brain tumors [1]. These tumors arise from glial cells, which are non-neuronal cells that provide support and nutrients to the neurons. Grade I tumors (e.g. pilocytic astrocytoma) and grade II tumors (e.g. oligodendroglioma) are considered as low-grade brain tumors, whereas grade III tumors (e.g. anaplastic astrocytoma) and grade IV tumors (e.g. glioblastoma) are classified as high-grade malignant brain tumors. Even though low-grade brain tumors generally possess more favorable characteristics concerning treatment possibilities and patient survival, they can still be lethal by progressing to higher grades of malignancy (e.g. low-grade diffuse astrocytoma can transform to anaplastic astrocytoma and glioblastoma) [2]. Gliomas account for 80% of malignant brain tumors, with the highly heterogeneous and highly infiltrating glioblastoma representing the largest and most-malignant subgroup (54% of gliomas) [1]. Regarding younger patients, gliomas

represent 55%, 40% and 34% of all brain tumors and 71%, 74% and 82% of malignant brain tumors in young children (age 0-14 years), adolescents (age 15-19 years) and young adults (age 20-34 years), respectively [1]. Glioblastoma can arise as *de novo* primary tumors, predominantly in older adults, or as secondary tumors that have developed over a period of several years from low-grade tumors, which occur predominantly in younger adults and are much less common than primary glioblastomas [3-4]. Both tumor types are morphologically and clinically indistinguishable [5], but show distinguishable gene alterations: primary glioblastoma are mainly characterized by overexpression or mutation of the epidermal growth factor receptor (*EGFR*), deletions and mutations in the phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*) and *p16* deletions [6-8], whereas secondary glioblastoma usually carry *p53* gene alterations and overexpression of platelet-derived growth factor receptor (*PDGFR*) [9]. Pediatric high-grade gliomas appear to be biologically and clinically distinct from adult high-grade gliomas, but show genetic alterations that are more similar to adult primary glioblastoma than adult secondary glioblastoma. Compared to high-grade gliomas in adults, *EGFR* amplification and mutation, *PTEN* mutations and *p53* alterations is relatively rare in high-grade gliomas in children [10-12]. However, just as in adult high-grade gliomas, *PDGFR* amplification was observed to similar extends and appeared to be mutually exclusive with *EGFR* amplification [10-11]. Quite contrary to this observation, *PDGFRA* has been shown to be the predominant target of focal amplification in pediatric high-grade gliomas [13], representing a useful marker in the distinction between high-grade gliomas in children and adults, and a possible therapeutic target. The standard treatment of glioblastoma includes surgical resection, radiotherapy and chemotherapy. Surgical resection is difficult due to the highly infiltrative nature of glioblastoma, rendering complete resection impossible [4]. After resection, state-of-the-art glioblastoma treatment consists of radiotherapy in combination with temozolomide treatment, leading to a median increase in survival of 2.5 months compared to

radiotherapy alone, and a median time to progression after treatment of 6.9 months [14]. An important marker in respect to temozolomide treatment is the O^6-methylguanine-DNA methyltransferase (MGMT) protein, a DNA repair enzyme that can repair DNA damages caused by DNA alkylating agents like temozolomide [15]. Significant survival benefits have been observed for patients carrying methylation of the *MGMT* promoter, which results in gene silencing [16]. Despite aggressive treatment, almost all glioblastoma eventually recur – 90% at the original site [4]. The median survival of low-grade astrocytoma patients is 5 years, but most patients die due to progression to high-grade astrocytoma, whereas the median survival for glioblastoma patients is one year [3].

Embryonal tumors, including medulloblastoma, are thought to arise from fetal or embryonal tissue due to alterations in normal developmental processes [17]. As mentioned above, medulloblastoma is the most common postnatal malignant brain tumor (children of 0-4 years) [1] and is considered as a grade IV malignant brain tumor [2]. These tumors can be further subdivided into classes of classic medulloblastoma, desmoplastic medulloblastoma, medulloblastoma with extensive nodularity, anaplastic medulloblastoma and large cell medulloblastoma [2]. Medulloblstoma account for 13% of all brain tumors in children younger than 14 years old [1] and mostly arise in the cerebellum [18]. After birth, the cerebellum is the only region in the brain that shows high rates of cell division, especially high proliferation of granule cell precursors [18], which is regulated by sonic hedgehog (SHH) signalling [19-20]. Therefore, genetic alterations in medulloblastoma include deregulations of the SHH signalling pathway [21-26], such as heterozygous loss of SHH receptor patched 1 (*PTCH1*) gene, alterations in the wnt signalling pathway [27-30], as well as *c-Myc* gene amplification and *c-Myc* mRNA overexpression [21]. Historically, medulloblastoma patients have been stratified into standard-risk and high-risk patients according to clinical factors. Patients older than three years of age with no evidence of

metastasis to other organs and none or small residual tumor post operation are considered as standard-risk patients, whereas patients who do not meet these criteria and show anaplastic histology are considered as high-risk patients [31]. Over the last decade, it became evident that stratification according to clinical parameters and histology alone is not sufficient to clearly decide on the most successful treatment. Further investigations led to the discovery of different molecular markers, which are better suitable for risk stratification and outcome prediction. These factors include for example TrkC expression and *c-Myc* mRNA expression [32], *c-Myc* amplification [33], overexpression of human epidermal growth factor receptor 2 [34], nuclear β-catenin and survivin expression [35], DNA copy-number aberrations of chromosomes 6q and 17q [36] and genes encoding for members of the histone deacetylase family (*HDAC5* and *HDAC9*) [37]. Standard treatment of medulloblastoma includes surgical resection followed by chemotherapy and radiotherapy. Compared to earlier treatments, today's treatments use lower doses of radiation by combination with different chemotherapeutic regimens [38-43], which is especially helpful for young patients since radiation therapy can cause deleterious side effects on the developing brain. This kind of combinational therapy as medulloblastoma treatment increases the 5-year event-free survival to 75% [44], even though almost half of the high-risk medulloblastoma patients die from tumor recurrence [45].

PI3K/AKT Signalling Pathway

The phosphoinositide 3-kinase (PI3K) pathway regulates several cellular processes such as cell survival, proliferation, growth, apoptosis and cytoskeletal rearrangement (reviewed in [46]). PI3Ks represent a family of protein and lipid kinases, which are divided into three classes (I-III) according to their sequence homology and *in vitro* lipid substrate specificity [47]. Class I PI3Ks are able to catalyze the phosphorylation of phosphoinositol (PI) on the inner leafted of the cell membrane. The main substrate of class I PI3Ks in cells is phosphatidylinositol-4,5-bisphosphate (PIP_2). Thus, class I PI3K activation leads to PIP_2 phosphorylation and results in the accumulation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3). Class I PI3K isoforms are further subdivided into two subgroups, depending on the receptors by which they are regulated. While class I_A binds to receptor tyrosine kinases (RTKs), class I_B is exclusively activated by G protein-coupled receptors [47-49]. Class I_A PI3Ks are heterodimers consisting of a p110 catalytic and a p85 regulatory subunit. The p110 catalytic subunits p110α, p110β and p110δ, which are encoded by *PIK3CA*, *PIK3CB* and *PIK3CD*, respectively, are highly homologous isoforms. *PIK3R1*, *PIK3R2* and *PIK3R3* encode the p85 isoforms which share a core structure that includes two Src homology 2 (SH2) domains and a p110 binding domain named inter-SH2 domain [50]. The adaptor subunit p85 is crucial for the activation of p110 by RTKs. Activation of the p85-p110 complex occurs either through direct interaction of the SH2 domain of p85 with phosphotyrosine residues on the RTK or through the adaptor proteins insulin receptor substrate 1 and 2 (IRS1 and IRS2) that couple to activated RTKs [51-52]. Class I_A PI3K can also be activated by the small GTPase Ras, through its Ras binding domain. This is of great importance, since oncogenic mutations in Ras lead to the constitutive activation of the PI3K signalling pathway. Similarly to class I_A, class I_B isoforms consist of a regulatory p101, p84 or p87PIKAP and a catalytic subunit p110γ.

There are three distinct class II PI3K isoforms: PI3KC2α, PI3KC2β and PI3KC2γ. In contrast to class I PI3K isoforms, they consist of only a single catalytic subunit and are able to catalyze the formation of phosphatidylinositol-3-monophosphate (PIP) and PIP_2, but not PIP_3. Although it is known that they are activated by RTKs, cytokine receptors and integrins, the consequences of this activation are not well understood [47]. Class III PI3Ks consist of a single member Vsp34, which can only phosphorylate the inositol ring of PI to PIP [47]. To date, there is much more published evidence about the role of class I_A PI3K in tumorgenesis. Therefore, it appears to have the most relevant function in this aspect within the PI3K family. Especially the class I_A PI3K p110α has become the focus of attention in the cancer research field over the past decade.

Activation of class I_A PI3Ks occurs upon ligand-mediated phosphorylation of different RTKs, such as insulin-like growth factor receptor 1 (IGF-1R), vascular endothelial growth factor receptor (VEGFR), EGFR or PDGFR in response to growth factor stimulation [53]. As a consequence, PIP_3 is generated and acts as a second messenger supporting the recruitment of the serine/threonine kinase AKT to the plasma membrane by binding to its pleckstrin homology domain [54-55] and activating several pathways downstream of AKT, as described below. PIP_3 levels in the cell are controlled by PI3K activity and by the lipid phosphatase PTEN, a 3-position phosphatase that converts PIP_3 back to PIP_2 [56-57]. These control mechanisms have antagonizing effects leading either to AKT activation or deactivation. PTEN is crucial for the regulation of the PI3K/AKT pathway, since there is no other related protein that compensates its loss of function. PTEN dysfunctions have been implicated in a wide range of cancer types, highlighting the importance of PTEN for the proper regulation of cell growth and proliferation [58].

Full activation of AKT is further achieved through phosphorylation of Thr308 by PDK1 and of Ser473 by the rapamycin-insensitive mTOR complex 2 (mTORC2) [59-62]. AKT represents a central node in the

intracellular signalling networks. Once activated, AKT phosphorylates numerous targets starting a series of signalling cascades involved in diverse cellular functions (see Fig. (1)).

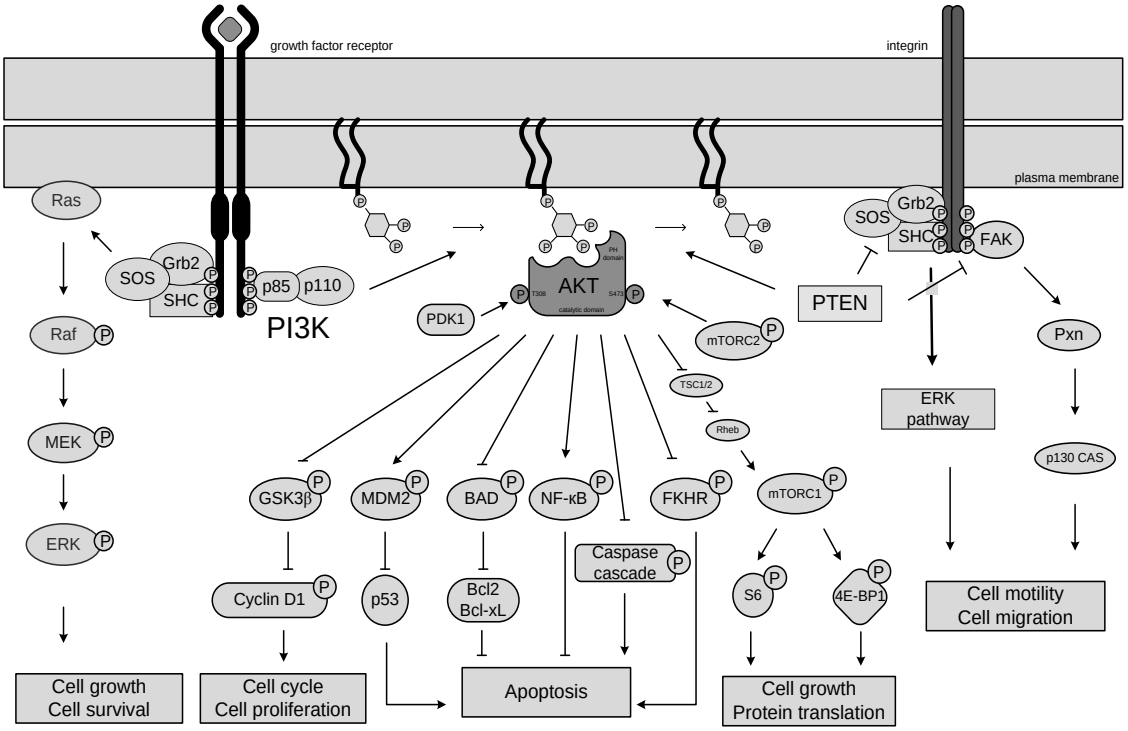

Fig. (1). Schematic representation of the PI3K/AKT signalling pathway. RTKs, EGFR and IGF-1R, are activated by autophosphorylation in response to growth factor binding. Upon activation of RTK, the Grb2-SOS complex binds to the receptor initiating the ERK pathway. On the other hand PI3K's regulatory subunit p85 dimerizes with its catalytic subunit p110. PI3K, when activated, catalizes the phosphorylation of PIP3 on the cell membrane, which binds subsequently to the PH domain of AKT. AKT phosphorylation is negatively regulated by the tumor suppressor PTEN. The recruitment of AKT to the cell membrane enables its phosphorylation by PDK1 and mTORC2. Following AKT activation, it initiates the activation of a cascade of protein kinases, involved in the regulation of cell growth, cell proliferation and apoptosis.

AKT inhibits apoptosis by direct phosphorylation of BAD [63], caspase-9 [64] and FKHR [65]. Inhibition of FKHR nuclear translocation prevents the activation of proapoptotic factors, such as Fas-Ligand and TRAIL. Additionally, AKT inhibits p53 dependent cell death by phosphorylation of MDM2 [66]. Besides the regulation of the anti-apoptotic pathway, AKT promotes cell cycle progression by the

inhibition of glycogen synthase kinase-3β (GSK-3β), leading to the accumulation of cyclin D1 via myc [67].

AKT also promotes cell growth by activation of the rapamycin-sensitive complex mTORC1 [68]. AKT phosphorylates the tuberous sclerosis complex 2 (TSC2) protein tuberin, inhibiting the ability of the TSC1-TSC2 complex to prevent Rheb activation [69-70]. This allows the activation of the mTORC1 complex, which in turn phosphorylates two regulators of protein translation and ribosomal biogenesis: p70 ribosomal S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) [71-72]. S6K phosphorylates the S6 protein of the small 40-S ribosomal subunit, ultimately leading to ribosome formation and thus protein synthesis. Phosphorylation of 4E-BP1 releases eIF4E, which subsequently forms complexes with several other initiation factors which control protein translation. mTOR is of special interest, since it is able to associate with two alternative partners, called raptor and rictor [62, 68, 72]. It operates in two places of the circuity, upstream of AKT as an mTOR-rictor complex (mTORC2) and downstream as an mTOR-raptor complex (mTORC1). This dual function gives mTOR powerful position in the cell. Hence, mTOR is able to regulate cell survival and proliferation by controlling AKT activity and cell growth by regulation of S6K and 4E-BP1 phosphorylation.

The importance of the PI3K/AKT signalling pathway in cancer is supported by the extensive evidence of deregulation of several components of the pathway in a wide spectrum of human cancers. Genetic analysis of human tumor samples performed over the past two decades have identified gain-of-function mutations and amplification in RTKs such as EGFR, PDGFR and IGF-1R [73-77], both leading to constitutively activated receptor signalling in cancer cells. Aberrant activation of the PI3K/AKT pathway can also occur through oncogenic mutations in the different PI3K isoforms. Mutations in the PI3K regulatory subunit p85 include deletions in the inter SH2 domain, which leads to PI3K activation independent of RTK signalling [78].

Genetic amplification or mutation of *PIK3CA* was identified in breast, colon, glioma, ovarian, gastric and cervical tumors [79-85] which makes p110α one of the most frequently mutated kinases in all tumor types [86]. Oncogenic mutations in all three *AKT* isoforms are also often observed in human cancers. While somatic mutations are more frequent in *AKT1*, gene amplification is mostly found in *AKT2* [87]. One of the most significant evidence of the involvement of PI3K signalling in cancer stems from studies of the *PTEN* gene. *PTEN* was first identified as a tumor suppressor gene in glioblastomas and breast cancer [58, 88] and was later widely implicated in various cancer types. *PTEN* inactivation occurs through several mechanisms such as loss of heterozygosity, loss of function mutations or through epigenetic silencing. *PTEN* is after *p53*, the second most commonly mutated tumor suppressor gene in cancer [89]. Further evidence of mutational studies indicates that PIP_3 phosphatase activity is responsible for the tumor-suppressor function of PTEN [56, 90], suggesting a key role of PIP_3 levels in carcinogenesis.

In this review, we will describe the role of key players of the PI3K/AKT pathway in the development of malignant brain tumors. Recent studies investigating the expression and alterations in this pro-survival signalling pathway will be summarized, while mainly focusing on the different PI3K isoforms and their antagonist PTEN. Further, we will discuss the progress made in the development of targeted therapies for the treatment of grade IV brain tumors. Emphasis will be put on pharmacological inhibitors against the pathway components EGFR, class I_A PI3K isoforms and mTOR. The different strategies to inhibit the pathway and the design of combinational therapies will be evaluated. Finally, we will discuss the future potential of inhibitors of the PI3K/AKT signalling pathway, as well as their issues and limitations as a novel anticancer strategy.

ALTERATIONS AND FUNCTIONS OF PI3K/AKT SIGNALLING PATHWAY COMPONENTS IN GRADE IV BRAIN TUMORS

Alterations in the pro-survival PI3K/AKT signalling pathway have been reported in a number of cancers, including cancers of the central nervous system. The most prominent genes altered include RTKs, mainly *EGFR* and its truncated version EGFRvIII, *PTEN* and *PIK3CA*, the gene encoding the catalytic subunit p110α of the class I_A PI3Ks. The focus of the discussion in this chapter is on alterations of the different PI3K isoforms and their antagonist PTEN. Alterations in EGFR in malignant brain tumors have been extensively reviewed previously [91-97] and would be beyond the scope of this review.

Most of the investigations concerning alterations in PI3K/AKT signalling in brain tumors have been performed with patient samples from glioblastoma, the most common and most malignant tumor of the central nervous system in adults. On the contrary, relatively few data are available in medulloblastoma, the most common malignant brain tumor in early childhood. An overview of the genetic alterations of major components of the PI3K/AKT pathway in these two tumor types is given in Fig. (2). This figure also indicates the percentage of glioblastoma and medulloblastoma tumor samples showing the corresponding gene alterations. The original studies reporting these data will be discussed below.

An investigation using general expression profiling in glioblastoma samples by cDNA microarrays determined that most of the genes altered in this type of cancer are involved in cell adhesion, signal transduction, cell cycle, apoptosis and angiogenesis [98].

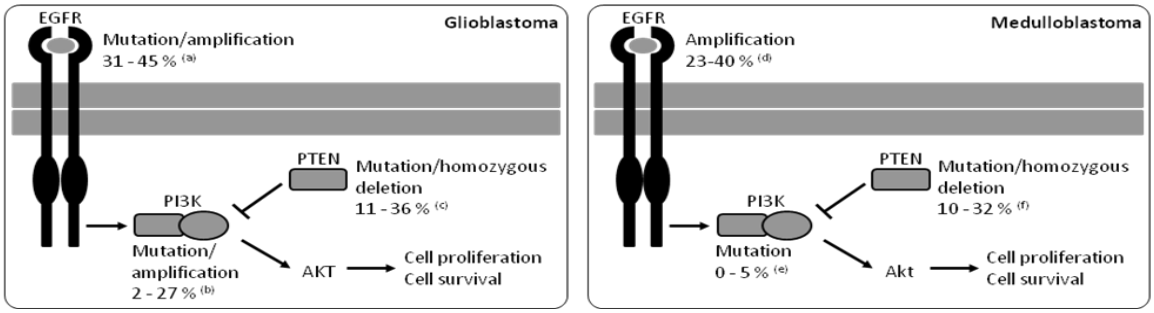

Fig. (2). Alterations in the PI3K/AKT signalling pathway in the most common malignant brain tumors of adults and young children, glioblastoma (left) and medulloblastoma (right), respectively. Percent of alterations detected in tumor samples are indicated. (a): [99-101, 133, 139], (b): [79, 81, 99-101, 118, 133, 139], (c): [58, 79, 99-101, 118, 139], (d): [103-104], (e): [79, 81], (f): [104-106].

The first study, that specifically investigated the genetic alterations in components of the PI3K/AKT signalling pathway in glioblastoma tumor samples, revealed genetic alterations in *PTEN* (loss of expression or homozygous deletion) and *EGFR* (gene amplification) in about one third of the patients [99]. Additionally, differential mRNA overexpression of the catalytic PI3K isoforms was detected [99]. An integrated analysis of 22 human glioblastoma samples by Parsons *et al*. revealed that alterations of genes of the PI3K/AKT pathway occur in around 50% of glioblastomas, including alterations in *EGFR* (37%), *PTEN* (30%), *PIK3CA* (10%) and *PIK3R1* (8%) [100].

The first comprehensive genomic characterization of 206 human glioblastoma samples was performed by The Cancer Genome Atlas (TCGA) Research Network and defined the pro-survival PI3K/AKT signalling pathway as one of the three core pathways in this cancer type, harboring at least one genetic event in 88% of glioblastoma tumor samples [101]. The other two core pathways defined by TCGA were the p53 signalling and the retinoblastoma (pRB) signalling pathways, which were found to be altered in 87% and 78% of glioblastoma samples, respectively [101]. Additionally, it has been observed that about three quarters of glioblastoma samples harbored alterations in all

three core pathways, suggesting that the deregulation of these pathways is a core requirement of glioblastoma pathogenesis [101].

An alternative way of investigating core pathways and driver genes in glioblastoma was performed by Cerami *et al*. using an automated network-based approach [102]. Taking into account that cellular networks contain functional modules and that tumors usually target specific modules that are critical for their growth, they used part of the original data created by TCGA as a starting point [102]. With this method they were able to identify many of the same driver gene candidates as the original TCGA approach (among these were *EGFR*, *PTEN*, *CDK4* and *MDM2*), defining the *PIK3R1* module as the largest and most densely interconnected network [102]. Additionally, their modules corresponded very closely to the three original TCGA core pathways and to the findings of the study by Parsons *et al*.

As mentioned above, studies on gene alterations in the PI3K/AKT signalling pathway in medulloblastoma are rare. Similar to observations in glioblastoma, these studies show alterations in *PTEN* and *EGFR* [103-105]. A study focusing on medulloblastoma revealed that activation of the PI3K/AKT signalling pathway is an important element of medulloblastoma cell proliferation and suggested that this activation probably represents a common event in medulloblastoma pathogenesis [106]. The authors argued that AKT activation is due to significantly reduced expression of *PTEN* mRNA and PTEN protein, rather than to mutations or homozygous deletions in the *PTEN* gene [106]. Another report, taking a closer look at PI3K signalling and the different PI3K isoforms in medulloblastoma, revealed that especially the catalytic subunit p110α is significantly upregulated in medulloblastoma samples compared to normal cerebellum [107]. This gives further evidence for the importance of PI3K/AKT signalling in medulloblastoma pathogenesis.

PTEN

Besides alterations in EGFR, mutation or loss of heterozygosity at chromosome 10q23 is a highly common phenomenon in cancers of the brain, especially in glioblastoma. The first description of the *PTEN* gene, which is located at this chromosome position, was performed by Li *et al*. [58]. The authors identified the PTEN protein as a protein tyrosine phosphatase and postulated that it acts as a tumor suppressor by antagonizing the action of PI3Ks. Further, *PTEN* mutations were commonly detected in primary glioblastomas (17-20% [58, 108]) but rarely in lower grade gliomas, postulating that mutations in this tumor suppressor arise rather late in tumorigenesis.

Cell culture experiments revealed several important functions of PTEN's lipid phosphatase activity in cell signalling, proliferation and survival. Next to the tumor suppressor function of PTEN, it was shown that loss of *PTEN* and expression of EGFRvIII act synergistically in the transformation of neural precursor cells, which induce tumors that resemble human glioblastomas [109]. Specifically, these cells were less dependent on epidermal growth factor (EGF) and insulin, showed increased migration and invasion, decreased susceptibility to apoptosis and enhanced formation of neurosperes and colonies in soft agar [109].

Not only has the lipid phosphatase activity of PTEN, which counteracts PI3K activity, been shown to play an important role in tumor cells, but also the protein phosphatase activity of PTEN has been linked to be involved in tumor cell functions. It has been shown that the protein and lipid phosphatase activities of PTEN are both required to suppress invasion, whereas either activity alone was sufficient to significantly inhibit proliferation [110]. Investigations on migrating glioma cells showed that integrin-dependent migration of these cells is regulated by the protein phosphatase activity of PTEN, but is independent of its lipid phosphatase function [111]. This integrin-dependent migration was independent of PI3K and AKT activity and the authors suggested that it is rather mediated by the action of the protein phosphatase of PTEN on

Src family kinase activity and downstream RAC1 GTPase activity [111]. Further, mutations in *PTEN* in glioblastoma cell lines have been shown to be associated with decreased radiosensitivity and sensitivity to temozolomide, which could be restored by introducing wild-type *PTEN* into these cells [112]. It was also suggested that PTEN has the capacity to induce the transcriptional activity of p53 in the tumor and stromal compartment, thereby suppressing the tumor-induced angiogenic response of glioblastoma cells *in vivo* [113]. It was proposed that the two tumor suppressor genes *PTEN* and *p53* are located on the same signalling axis and coordinate the control of growth and angiogenesis in brain tumor cells [113].

Supporting results that were obtained from cell culture experiments, analysis of glioblastoma patient samples showed that loss of tumor suppressor protein PTEN is highly correlated with expression levels of phosphorylated AKT and AKT activity [114-115]. Additionally, it was found that activation of AKT is significantly correlated with levels of phosphorylated mTOR, activation of the family of Forkhead transcription factors and ribosomal protein S6 phosphorylation [114]. A significant inverse correlation was determined between PTEN protein expression and glioma grade, showing significantly decreased PTEN expression in grade IV glioblastomas compared to lower grade gliomas and non-tumor brain tissue [116]. Additionally, a significant correlation between PTEN protein expression and the survival time of glioblastoma patients was detected [115], favoring patients with PTEN expression.

PTEN promoter methylation might serve as a marker to distinguish between different grades of gliomas. It was shown that methylation of the *PTEN* promoter was common in grade II and III astrocytomas, oligodendrogliomas, oligoastrocytomas and secondary glioblastomas, whereas it was rarely detected in primary glioblastomas and not detected at all in non-tumor brain samples [117]. This gives further evidence to the evolution of secondary glioblastoma from lower grade gliomas, indicating that secondary glioblastomas would share some

characteristics like their low-grade precursors. It was speculated that PI3K activation via *PTEN* alteration is different in primary than in secondary glioblastomas: promoter methylation and loss of heterozygosity of *PTEN* might be the mechanisms in secondary glioblastoma, whereas mutation plus methylation or loss of heterozygosity plus methylation might be the mechanisms in primary glioblastomas [117]. Further speculation about the PI3K/AKT signalling pathway activation in secondary glioblastoma includes the possibility of *PIK3CA* gain-of-function mutations rather than *PTEN* mutations [79]. *PTEN* mutations are commonly found in primary glioblastomas and are rather uncommon in secondary glioblastomas, whereas it appears to be the opposite for mutations in *PIK3CA*: no *PTEN* mutations were found in tumors with mutations in *PIK3CA* [79]. This might indicate that mutations in *PTEN* and *PIK3CA* are mutually exclusive in malignant glioma.

In primary medulloblastoma samples, it was observed that *PTEN* mRNA expression was significantly lower when compared to normal cerebellar tissue, independently of medulloblastoma histological subtype. In addition, PTEN protein was not detectable by immunohistochemical staining in medulloblastoma samples when compared to control tissue [106]. *PTEN* mRNA was detected in all medulloblastoma cell lines investigated and indicates that homozygous deletion of *PTEN* does not seem to be a common mechanism in medulloblastoma [106]. Rather, the authors reported that 50% of the samples harbor *PTEN* promoter methylation, suggesting that alterations in transcription may be involved in PTEN regulation that leads to activation of the PI3K/AKT signalling pathway [106]. On the contrary, a different study found that the PTEN protein was expressed at higher levels in medulloblastoma samples and cell lines as compared to control samples [107].

PIK3CA and Genes Encoding other PI3K Isoforms

As mentioned above, differential mRNA expression levels of PI3K isoforms have been detected in one of the first investigations of alterations in the PI3K/AKT signalling pathway in glioblastoma. Gene amplification of *PIK3C2B* (6%) but not of *PIK3CD* and *PIK3CA* was detected, while over-expression of *PIK3CD* mRNA (6%) was present [99]. Differential levels of the different class I_A PI3K isoforms have also been detected in medulloblastoma patient samples. Significant overexpression of *PIK3CA* gene in medulloblastoma tumor samples was found compared with normal cerebellum, whereas no significant differences in expression was observed for the remaining class I_A PI3K isoforms (catalytic and regulatory subunits) [107]. Compared to normal brain tissue, protein overexpression of the PI3K catalytic isoform p110α was observed in human medulloblastoma samples (74%) as well as high basal expression of phosphorylated AKT and phosphorylated ribosomal protein S6 [107]. It was shown that PI3K p110α promotes medulloblastoma cell survival, activates signalling through AKT, is important in protecting medulloblastoma cells against chemotherapy-induced cell death and supports the migratory capacity of these cells [107].

Mutations in the *PIK3CA* gene in brain tumor samples have been reported in different studies and to different extents. A first investigation by Samuels *et al*. in 2004 found high frequencies of *PIK3CA* mutations in human cancers, including glioblastoma (27%), but no mutations in medulloblastoma samples [81]. On the contrary, mutations in the *PIK3CA* gene were detected in only 5% of glioblastoma samples, as well as in 5% of medulloblastoma patient samples, using a large scale mutational analysis of different brain tumor types by Broderick *et al*. [79]. The differences in the percentage numbers between these two studies might be due to the fact that in the first study, the whole *PIK3CA* gene was investigated, whereas in the latter study only exons 9 and 20 – encoding for the helical and catalytic domain, respectively – of the gene were investigated. However,

Samuels *et al*. observed that most of the mutations in *PIK3CA* were clustered in the helical and catalytic domain [81]. Nevertheless, both studies reached the conclusion that *PIK3CA* mutations occur late in tumorigenesis, since *PIK3CA* mutations have been shown in late stage colon cancer [81] and more frequently in secondary glioblastoma than in primary glioblastoma [79]. Interestingly, it has been stated by Broderick *et al*. that no significant *PIK3CA* gene amplification or increase in gene expression was detected in glioblastoma and medulloblastoma samples [79]. This suggests that the higher activation of PI3K p110α in brain tumors is not due to gene amplification or overexpression but rather results from mutations in the *PIK3CA* gene. A third study, by Hartmann *et al*. [118], examined all coding exons of the *PIK3CA* gene and observed mutations in 7% of glioblastoma patient samples, rather giving support to the study by Broderick *et al*. The authors also showed that the detected mutations clustered together in specific regions of the gene. Apart from malignant brain tumors, this effect has also been observed in other human cancer types [80, 119-124] and gives further support to the concept of somatic "hot spot" mutations. It was demonstrated, that the "hot spot" mutant *PIK3CA* H1047R (catalytic domain) conferred more lipid kinase activity in immunoprecipitated PI3K complexes than the wild-type protein [81], suggesting that the catalytic isoform p110α functions as an oncogene in human cancers. This observation was further supported by other research groups and in other cancer types, showing that the conserved "hot spot" mutations in *PIK3CA* are indeed able to increase kinase activity and that they possess oncogenic potential [81, 122, 125-130]. As already observed by Broderick *et al*., *PIK3CA* and *PTEN* mutations seem to be mutually exclusive, because tumors harboring mutations in *PIK3CA* show a lack of mutations in *PTEN* [79, 118]. Therefore, it can be speculated that mutations in *PIK3CA* provide an alternative mechanism to activate PI3K/AKT signalling. However, as mentioned before, *PIK3CA* mutations appear at relatively low rates and might thus, compared to *PTEN*, play only a minor role in glioblastoma pathogenesis.

The crystal structure of the adaptor-binding domain of the catalytic p110α isoform (bovine) in complex with the inter-Scr homology 2 (iSH2) domain of the regulatory PI3K p85α isoform (human) was recently solved by Miled *et al*. [131] and led to a model of the mechanism of PI3K p110α activation by mutations in *PIK3CA*. The authors found three cancer-specific mutations that were located in the adaptor-binding domain, but none of these residues was located at the interface between the PI3K p110α adaptor-binding domain and the PI3K p85α iSH2 domain [131]. It was rather observed that there was a highly conserved adaptor-binding domain surface patch outside this conserved interface, where two of these mutations were located and represented a plausible docking site for other domains of the complex [131]. Mutations in this surface patch may reorient the adaptor subunit or even change the orientation of the enzyme on the membrane [131]. Increased PI3K p110α activity probably results from a destruction of charge-charge interactions between the PI3K p110α and PI3K p85α subunits, which is caused by mutations in the helical domain of PI3K p110α and results in the inability of a minimal PI3K p85α fragment to inhibit PI3K p110α's catalytic activity [131].

A more recent determination of the crystal structure of PI3K p110α (all five domains) in complex with PI3K p85α (only N-terminal SH2 and iSH2 domains) by Huang *et al*. [132] made it possible to determine the effects of *PIK3CA* mutations in all PI3K p110α domains. The surface patch of the adaptor-binding domain mentioned above was shown to be in hydrogen-bonding distance with residues of the PI3K p110α kinase domain, suggesting that mutations in this patch might result in conformational changes of the kinase domain, which would probably alter enzymatic activity [132]. Another cancer-specific mutation location in the C2 domain of PI3K p110α was shown to be within hydrogen bonding distance of the PI3K p85α iSH2 domain residues, suggesting that this mutation may disrupt the interaction between these two domains and would presumably alter the regulatory effect of p85α on p110α [132]. "Hot spot" mutations in the helical domain cause a

charge reversal and, consistent with Miled *et al*., presumably abrogate the inhibitory effect of the p85α nSH2 domain by modifying the orientation of the p85α nSH2 domain to the helical and kinase domains of p110α [132]. Alterations in the kinase domain of PI3K p110α, like the "hot spot" mutant *PIK3CA* H1047R, most likely have a direct effect on the conformation of the activation loop and would therefore change the interaction with PI substrates [132].

Somatic alterations in *PIK3R1* have been much less frequently reported than alterations in *PIK3CA*, and consist of truncations and deletions or mutations in the iSH2 domain [101, 132]. The first report of an alteration in the regulatory PI3K p85α subunit gene has been reported by Mizoguchi *et al*., namely a 9-base pair deletion, along with frequent balanced copy number increases of both *PIK3CA* and *PIK3CD* [133]. The alterations in *PIK3R1* result in the destabilization of the PI3K p85α iSH2 domain, which is important for the contact with the PI3K p110α C2 domain [132]. These mutations would therefore relieve the inhibitory effect of PI3K p85α on PI3K p110α and seem to have equivalent effects as mutations in the p110α C2 domain. PI3K p85α regulatory subunit mutants, with mutations in the iSH2 domain but intact p110-binding site, do not affect the stabilization of the p110 subunits but impair the ability of p85α to negatively regulate p110 isoform activity, leading to promotion of cell survival and anchorage-independent growth, as well as to increased levels of phosphorylated AKT when compared to wild-type PI3K p85α cells (in a PI3K p110-activity-dependent manner) [134]. It has also been shown that these mutant PI3K p85α/p110(α/β or δ) holoenzymes can be effectively inhibited by p110(α/β or δ) inhibitors, indicating that pan-p110 inhibitors are likely to be efficacious in patients with PI3K p85α mutations [134].

As can be seen above, most of the studies on PI3K alterations in brain tumors have been focusing on class I_A PI3K ioforms, especially PI3K p110α. Isoforms of class II PI3Ks have not been studied that intensively, but PI3KC2β has lately been detected to possibly play an

important role in glioblastoma resistance to erlotinib, an EGFR tyrosine kinase inhibitor. It has been demonstrated that the expression of *PIK3C2B* gene correlated significantly with glioblastoma cellular resistance towards erlotinib [135], and PI3KC2β might therefore as well represent a possible therapeutic target in glioblastoma.

The PI3K/AKT signalling pathway has also been linked to other important features of gliomas, such as radioresistance, migration and clinical outcome. For example, glioblastoma cell lines with activated AKT due to loss of *PTEN* expression have been shown to be more resistant against radiation therapy after serum starvation [136]. PI3K activity has also been shown to be necessary for AKT activation in actively migrating glioblastoma cells, where phosphorylated AKT was mainly located at the leading edge of the migrating cells [137]. When migration was suppressed in glioma cells, the cells were more prone to apoptosis, suggesting that the suppression of apoptosis by PI3K activation is accompanied by the acquisition of migration [137]. Components of the PI3K/AKT signalling pathway have also been shown to correlate well with proliferating glioma cells and tumor grade of human gliomas (AKT2 [138]), and clinical outcome of patients with glioblastoma (*PIK3CD* [98]). Another gene related to the PI3K/AKT signalling pathway is *PIKE* and it was shown to be amplified in 12%-19% of glioblastoma samples [102, 139]. PIKE is a PI3K enhancer, which binds to and enhances AKT kinase activity in a GTP-dependent manner [140]. This gene was shown to be co-amplified with the adjacent CDK4 gene in primary glioblastoma samples [139] and has additionally been identified to be part of the *PIK3R1* module in the automated network analysis by Cerami *et al*. [102]. This indicates that *PIKE* represents a putative oncogene and an activator of the PI3K/AKT signalling pathway.

Overall, genetic alterations in PI3K subunit genes seem to occur in human glioblastoma at a lower rate than originally suspected and they are much less common than alterations in the *PTEN* gene. Nevertheless, they represent a PTEN deregulation-independent

alternative mechanism for the activation of the PI3K/AKT signalling pathway and render the different PI3K isoforms as potential clinical targets.

TARGETING THE PI3K/AKT SIGNALLING PATHWAY IN GRADE IV BRAIN TUMORS

Due to the relatively high incidence of alterations in PI3K/AKT signalling in cancer, this pathway represents an attractive target for the treatment of malignant brain tumors. As has been discussed above, PI3K/AKT pathway activation occurs through different mechanisms, involving EGFR overactivation by amplification or mutation, gain-of-function mutations in *PIK3CA*, as well as loss-of-function of *PTEN* tumor suppressor gene. Consequently, activation of the PI3K signalling network appears to be an obligatory event in glioblastoma progression. It seems feasible that counteracting the effects of *PTEN* loss and/or EGFR overactivation in brain tumors can have therapeutic advantages. Indeed, a few studies have shown that re-expression of PTEN inhibits the growth and transforming ability of glioma cells, and sensitizes them to chemotherapeutic agents such as etoposide [141-142]. Restoration of PTEN expression in *PTEN* null cells is not a clinically applicable approach at present. Instead, small molecule inhibitors targeting the components of the PI3K/AKT signalling pathway represent a more promising strategy. Importantly, it has been shown in a recent study that patients with heavily-pretreated, diverse, advanced cancers and *PIK3CA* mutations display significantly higher response rates to treatment with inhibitors targeting the PI3K/AKT signalling pathway than patients without *PIK3CA* mutations [143]. An overview of small molecule inhibitors of the PI3K/AKT signalling pathway that are currently used in clinical trials are shown in Fig. (**3**) and Fig (**4**), indicating their corresponding point of action and their chemical structure, respectively.

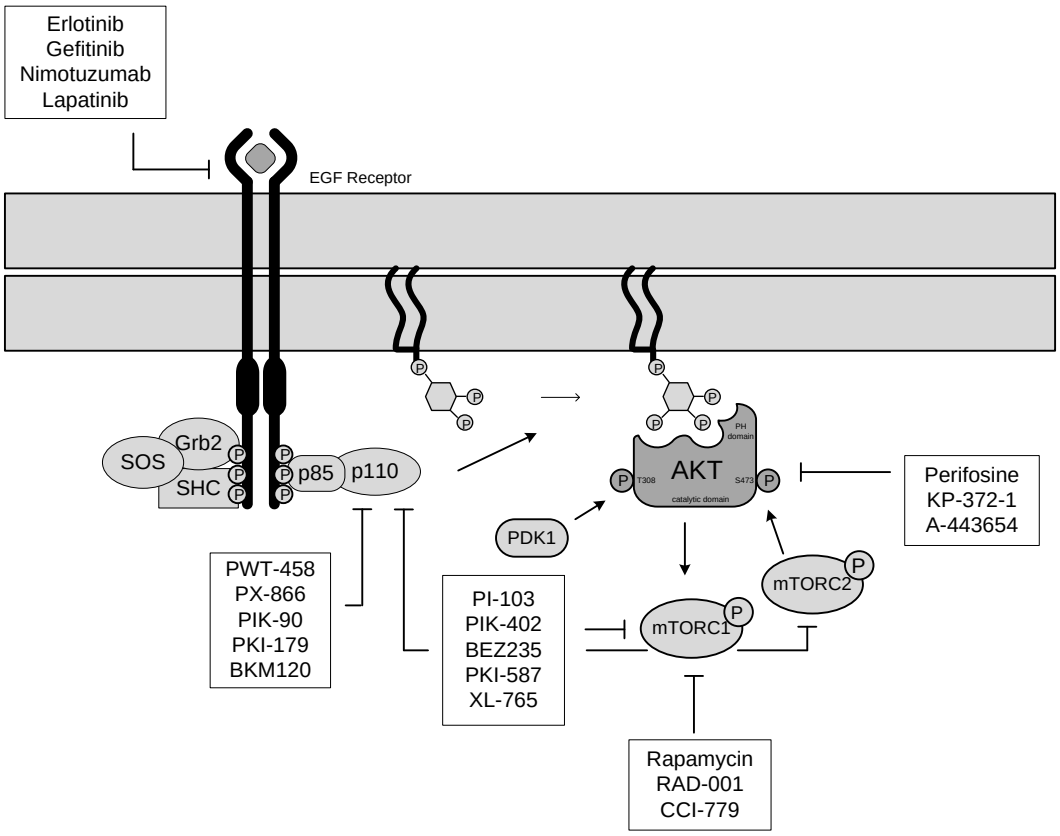

Fig. (3). Overview of small molecule inhibitors targeting the PI3K/AKT signalling pathway. The components EGFR, PI3K, AKT and mTOR are considered as potential therapeutic targeting sites in the pathway.

EGFR Inhibitors

EGFR mutation and amplification represent the most prominent genetic alterations in malignant brain tumors and identified this RTK as a suitable target for the treatment of this type of tumor. The use of small molecule inhibitors that target the EGFR generated great excitement in the first instance. Currently, the EGFR tyrosine kinase inhibitors gefitinib and erlotinib are being tested in a number of clinical trials [95, 144-153]. Although these drugs were well tolerated by patients, clinical responses have been disappointing and rarely durable. Combinatorial treatments of EGFR inhibitors with chemotherapy and/or radiotherapy have yielded better, although still modest results. An overview of ongoing clinical trials in the different types of brain tumors is summarized in Table 2 (information in this table has been retrieved

from [154]). The poor responses to EGFR inhibition can be partly explained, by the fact that mutations in *PTEN* or in *PIK3CA* effectively uncouple EGFR from PI3K/AKT signalling. This hypothesis is supported by a few studies. Work by Mellinghoff *et al*. analyzed the response of 49 patients with recurrent malignant gliomas to EGFR inhibition in correlation with the expression of EGFR, its constitutively active mutant EGFRvIII and PTEN. This study demonstrated that expression of EGFRvIII sensitized tumors to EGFR inhibitors, only if *PTEN* was intact [155]. Similar results were obtained in an animal xenograft model, which showed that erlotinib-sensitive glioblastoma xenografts shared the following molecular characteristics: wild-type *PTEN* and increased EGFR expression [156]. A subsequent study performed in a cohort of 41 glioma patients found that response to erlotinib was associated with high EGFR expression levels and low phosphorylation levels of AKT [146]. None of the patients whose tumors had high levels of activated AKT responded to erlotinib. Further studies by Fan *et al*. showed that EGFR signalling through PI3K, in particular through mTOR, is critical for mediating the tumor cell response to EGFR inhibitors [157]. Together, these findings highlight the importance of an intact PI3K regulation in the responsiveness of tumors to EGFR inhibitors. Targeting signalling components downstream of EGFR might therefore offer a more efficient strategy to inhibit cell survival.

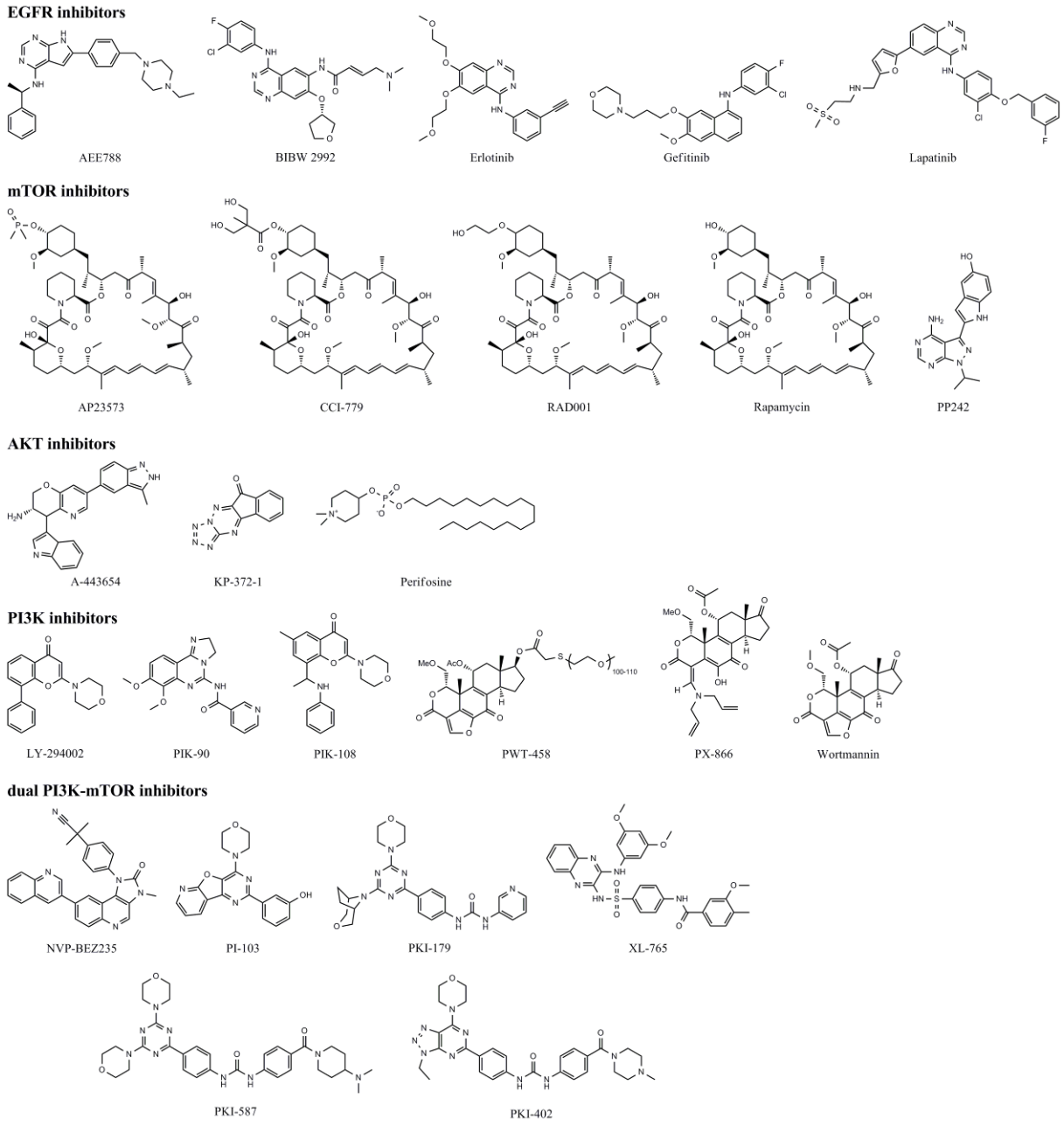

Fig. (4). Chemical structures of small molecule inhibitors targeting the PI3K/AKT signalling pathway. Structures were obtained from [174, 188, 206-207, 213, 220, 227-233].

Table 2. Ongoing clinical trials in brain tumors. Information about clinical trials has been retrieved from [154].

EGFR inhibitors in clinical trials

Drug	Targets	Patient group	Phase	State	Trial ID	Ref.
Erlotinib	EGFR	GBM	II	completed	NCT00337883	
	EGFR	Glioblastoma, other solid tumors	I	completed	NCT00030498	[148]
	EGFR	GBM	I, II	completed	NCT00045110	[149]
	EGFR	GBM, Anaplastic Astrocytoma, Anaplastic Oligodendroglioma, Glioblastoma, Gliosarcoma, Mixed Glioma	I	recruiting	NCT01103375	
	EGFR	GBM	II	unknown	NCT00086879	
	EGFR	GBM	I	unknown	NCT00227032	
	EGFR	GBM, Anaplastic Astrocytoma	I, II	recruiting	NCT00301418	
	EGFR	GBM	II	unknown	NCT00054496	
Gefitinib	EGFR	GBM	II	completed	NCT00250887	
	EGFR	GBM	II	completed	NCT00014170	[153]
	EGFR	GBM	II	completed	NCT00016991	[151]
	EGFR	malignant CNS tumors	II	completed	NCT00025675	[152]
Nimotuzumab	EGFR	GBM	II	completed	NCT00561873	
BIBW 2992	HER2	brain tumors, metastasis in brain	II	completed	NCT00875433	
Lapatinib	HER2	GBM	II	completed	NCT00099060	
	HER2	Glioblastoma, Gliosarcoma	I, II	completed	NCT00103129	
Combination therapy						
Erlotinib + bevacizumab	EGFR + VEGF	Glioblastoma, Gliosarcoma	II	ongoing	NCT00671970	
Erlotinib + radiation	EGFR	low-, high-grade glioma	I, II	ongoing	NCT00124657	
Erlotinib + sorafenib	EGFR + Raf-1, VEGFR, PDGFR	GBM	II	completed	NCT00445588	
Erlotinib + temozolomide, radiation	EGFR	GBM	II	ongoing	NCT00274833	
	EGFR	Glioblastoma, Gliosarcoma	II	ongoing	NCT00039494	
	EGFR	Glioblastoma, Gliosarcoma	II	unknown	NCT00187486	
Erlotinib + bevacizumab, radiation, temozolomide	EGFR + VEGF	Glioblastoma, Gliosarcoma	II	recruiting	NCT00720356	
Erlotinib + dasatinib	EGFR + SCR, ABL	GBM	I	unknown	NCT00609999	
Gefitinib + irinotecan	EGFR	Medulloblastoma, Glioblastoma	I	recruiting	NCT00132158	
Gefitinib + radiation	EGFR	GBM	II	completed	NCT00238797	[234]
	EGFR	GBM	I, II	unknown	NCT00052208	
Gefitinib +	EGFR	malignant primary glioma	I	completed	NCT00027625	

temozolomide						
Cetuximab + temozolomide, radiation	EGFR	malignant glioma	I, II	unknown	NCT00311857	
Cetuximab + bevazicumab, irinotecan	EGFR + VEGF	malignant glioma	II	completed	NCT00463073	
Nimotuzumab + temozolomide, radiation	EGFR	GBM	III	ongoing	NCT00753246	
BIBW 2992 + temozolomide, radiation	HER2	Glioblastoma	I	recruiting	NCT00977431	
BIBW 2992 + temozolomide	HER2	GBM	II	ongoing	NCT00727506	
Panitumab + Irinotecan	EGFR	GBM	II	recruiting	NCT01017653	

mTOR inhibitors in clinical trials

Drug	**Targets**	**Patient group**	**Phase**	**State**	**Trial ID**	**Ref.**
AP23573	mTORC1	Glioblastoma, malignant CNS tumors	I	completed	NCT00087451	
CCI-779 (Temsirolimus)	mTORC1	GBM	II	completed	NCT00016328	[163]
	mTORC1	GBM	I, II	completed	NCT00022724	[162]
RAD-001 (Everolimus)	mTORC1	GBM, Astrocytoma	II	terminated	NCT00515086	
Rapamycin (Sirolimus)	mTORC1	GBM	I, II	ongoing	NCT00509431	
	mTORC1	GBM	I, II	completed	NCT00047073	[167]
Combination therapy						
CCI-779 + doxorubicin	mTORC1	Glioblastoma, malignant solid tumors	I	ongoing	NCT00703625	
	mTORC1	Glioblastoma, malignant solid tumors	I	ongoing	NCT00703170	
CCI-779 + erlotinib	mTORC1 + EGFR	Glioblastoma, Gliosarcoma	I, II	ongoing	NCT00335764	
CCI-779 + vandetanib	mTORC1	GBM	I	ongoing	NCT00112736	
CCI-779 + radiation	mTORC1	Glioblastoma	II	recruiting	NCT01019434	
CCI-779 + sorafenib	mTORC1	GBM	I, II	recruiting	NCT00329719	
CCI-779 + temozolomide	mTORC1	brain tumors	I	completed	NCT00784914	
CCI-779 + temozolomide, radiation	mTORC1	GBM	I	ongoing	NCT00316849	
RAD-001 + radiation	mTORC1	GBM	I, II	recruiting	NCT01062399	
RAD-001 + temozolomide	mTORC1	GBM	I	ongoing	NCT00387400	
RAD-001 +	mTORC1	Glioblastoma	I, II	recruiting	NCT00553150	[235]

temozolomide, radiation						
RAD-001 + temozolomide, radiation, bevacitumab	mTORC1 + VEGF	Glioblastoma	II	ongoing	NCT00805961	
RAD-001 + AEE788	mTORC1 + EGFR, VEGFR	GBM	I, II	unknown	NCT00107237	
RAD-001 + gefitinib	mTORC1 + EGFR	GBM	I, II	unknown	NCT00085566	
RAD-001 + gleevec, hydroxyurea	mTORC1 + PDGFR, BCR-Abl	Glioblastoma, Gliosarcoma	I	unknown	NCT00613132	
Rapamycin + erlotinib	mTORC1 + EGFR	Glioblastoma, Gliosarcoma	II	completed	NCT00672243	
Rapamycin + vandetanib	mTORC1 + VEGFR2	GBM	I	recruiting	NCT00821080	

PI3K inhibitors in clinical trials

Drug	**Targets**	**Patient group**	**Phase**	**State**	**Trial ID**	**Ref.**
PX-866	pan PI3K	Glioblastoma	II	recruiting	NCT01259869	
BKM120	pan-PI3K	Glioblastoma	II	recruiting	NCT01339052	
XL-147	pan-PI3K	Glioblastoma	I	recruiting	NCT01240460	
PKI-179	PI3K, class I_A	malignant solid tumors	I	terminated	NCT00997360	
PKI-587	PI3K, class I_A mTORC1 mTORC2	Glioblastoma	I	recruiting	NCT00940498	
Combination therapy						
XL-765 + temozolomide	PI3K, class I_A mTORC1 mTORC2	Glioblastoma	I	recruiting	NCT00704080	
BKM120 + bevacizumab	pan-PI3K, VEGF	GBM	I/II	not yet recruiting	NCT01349660	

AKT inhibitors in clinical trials

Drug	**Targets**	**Patient group**	**Phase**	**State**	**Trial ID**	**Ref.**
Perifosine	AKT	malignant glioma	II	ongoing	NCT00590954	
Combination therapy						
Perifosine + CCI-779	AKT + mTORC1	GBM	I, II	recruiting	NCT01051557	
Perifosine + CCI-779	AKT + mTORC1	medulloblastoma, pediatric solid tumors	I	recruiting	NCT01049841	

mTOR Inhibitors

The most extensively used drugs that target mTOR are rapamycin (sirolimus) and its analogs, CCI-779 (temsirolimus) and RAD-001 (everolimus). Numerous studies have demonstrated the efficacy of mTOR inhibitors in *in vitro* and *in vivo* models (reviewed in [158-159]). Unfortunately, clinical studies could not reproduce the promising results of these drugs in laboratory models in the treatment of glioma patients [160-163]. Considering that cancer cells favor the accumulation of multiple genetic alterations, several resistance mechanisms may be present. Therefore, single agent treatments are unlikely to have an impact on overall tumor progression. For example, it has been found that rapamycin leads to an activation of ERK1/2 and MEK1/2 in glioblastoma, while the combined inhibition of MEK1/2 and mTOR leads to significantly suppressed cell proliferation compared to mTOR inhibition alone. These observations suggest that the limited benefit of rapamycin and its analogs can be explained by a feedback between mTOR and MAPK signalling [164]. The failure of EGFR inhibitors to impact on mTOR signalling in glioma suggested a rationale to combine inhibitors of EGFR and mTOR. Preclinical studies confirmed this hypothesis providing evidence that inhibitors of EGFR and mTOR had synergistic anti-tumor effects in glioblastoma [160, 165]. One of the first completed clinical trials reported by Reardon *et al*. aimed at investigating the anti-tumor effects of gefitinib and sirolimus in patients with recurrent malignant glioma. While only 6% of the patients achieved a partial response, 38% achieved stable disease [166]. Similar results were reported by Doherty *et al*. The combinatorial treatment of gefitinib or erlotinib with sirolimus led to a partial response in 19% of the patients and a six-month progression-free survival of 25% [161]. This combinational approach was only efficient in a subgroup of patients, highlighting the importance of patient stratification based on molecular characteristics of the tumor.

A subsequent phase I clinical trial aimed at analyzing responses of glioblastoma patients to rapamycin treatment in dependence of the

molecular features of the tumors [167]. Rapamycin was able to significantly inhibit mTOR activity within tumors in a subset of patients and this correlated with reduced proliferation. Surprisingly, in half of the patients rapamycin led to a shorter time to tumor progression, which correlated with increased levels of phosphorylated AKT. This effect can be in part explained because mTOR inhibition by rapamycin not only leads to inactivation of mTORC1 dependent cell growth signalling, but also suppresses negative feedback loops, that attenuate PI3K signalling in response to mTORC1 activation. Among them, activation of S6K by mTORC1 initiates a feedback mechanism by which S6K directly phosphorylates IRS1 causing its inactivation and consequently, decreasing AKT activation. Thus, rapamycin treatment results in mTORC1-mediated S6K inactivation and thereby loss of feedback inhibition that leads to reconstitution of AKT activation, explaining the observed increase in tumor cell survival [167-168]. Secondly, inhibition of mTORC1 by rapamycin does not efficiently induce dephosphorylation of 4E-BP1 in most cellular contexts [169]. Rapamycin does not have the same affinity for mTORC2 as for mTORC1 [170]. As mentioned above, mTORC2 directly phosphorylates AKT promoting its activation. At low rapamycin concentrations, mTORC2 complexes stay uninhibited and may become hyperactive, contributing to AKT activation [171-172].

In order to overcome these limitations, a new generation of ATP-competitive active site mTOR inhibitors is under development. Contrary to rapamycin and its analogs, which inhibit mTOR through allosteric interactions, active site mTOR inhibitors target the catalytic site of both mTORC1 and mTORC2 (reviewed in [158]). Several independent studies are currently proving the anti-proliferative capacities of the different active site mTOR inhibitors, with the expectation that targeting both mTOR complexes, downstream and upstream of AKT, will have a more dramatic effect on carcinogenesis. It has been published that active site mTOR inhibitors more potently inhibit cell growth, cell cycle progression and protein translation in

several cell lines compared to rapamycin and its analogues [171, 173-176]. Furthermore, active site mTOR inhibitors strongly inhibit 4E-BP1 phosphorylation. An exciting recent study showed that PP242, an active site mTOR inhibitor, has a strong anti-tumor effect in T-cell lymphomagenesis *in vivo* with no noticeable toxicity. This effect was mediated through inhibition of mTORC1-dependent 4EBP-eIF4E hyperactivation [177]. Although there are no published studies about the effects of ATP-competitive active site mTOR inhibitors in brain tumors yet, great expectations for their anti-proliferative potential have emerged from the successful results in other cancer types.

A note-worthy disadvantage of active site mTOR inhibitors is that they do not overcome the problematic loss of feedback inhibition of AKT observed after rapamycin-mediated mTORC1 inhibition. Any type of inhibition of mTORC1 will disrupt the negative feedback mechanisms. Targeting specifically mTORC2 could thereby be a better approach, since it would not only directly block AKT phophorylation, moreover it would not perturb the mTORC1-dependent feedback loops. Although specific mTORC2 inhibitors do not exist yet, it is speculated that they could have good therapeutic responses [170].

Another promising approach to efficiently block abnormal activation of the PI3K/AKT pathway is targeting components upstream of mTOR. For example, simultaneous targeting of mTOR and AKT in glioblastoma cells has been shown to efficiently downregulate the PI3K signalling pathway activation. Furthermore, this combination resulted in decreased cell proliferation and increased cell death in *in vivo* and *in vitro* experiments, independent of the PTEN-status [178]. As will be discussed further below, a class of kinase inhibitors with dual specificity against PI3K and mTOR was shown to have strong anti-tumor effects in several cancer types, including brain tumors (reviewed in [179]).

AKT Inhibitors

AKT is a central node in the complex network of signalling pathways [53] and its role has been extensively investigated in many cancer types [180]. *In vivo* studies have shown the importance of AKT signalling for glioblastoma maintenance in the context of activated Ras, as expression of AKT negatively correlated with increased survival of glioblastoma tumor-bearing mice [181]. Although there are few preclinical reports about the pharmacological targeting of AKT in brain tumors, there is evidence that inhibition of AKT has anti-proliferative and radiosensitizing effects [182-185]. In the glioma cell lines U87MG and U251, inhibition of AKT by KP-372-1 and KP-372-2 resulted in cell growth inhibition through the induction of apoptosis [182]. Consistent with this finding, AKT was shown to have a protective role in TRAIL-induced apoptosis [183]. The small molecule AKT inhibitor A-443654 recently showed strong anti-tumor effects *in vivo* and *in vitro*, it significantly extended survival rates in a rat intracranial model of glioblastoma compared to control animals [184]. The radiosensitizing potential of AKT inhibitors has been demonstrated by several studies [109, 186-187]. It was shown that ionizing radiation induced AKT activation in a group of glioblastoma cell lines. AKT inhibitors increased the radiosensitivity in these cells, suggesting that AKT is activated as part of a feedback mechanism mediating radioresistance [109].

One of the most prominent AKT inhibitor is probably perifosine, a lipid-based AKT inhibitor that interacts with the pleckstrin homology domain of AKT preventing its translocation to the plasma membrane. Perifosine is currently being tested in a series of clinical trials in different cancers (reviewed in [188-189]). Although perifosine was generally well-tolerated by patients, tumor response was variable between different cancer types. While in sarcoma and waldenstrom macroglobulinemia patients perifosine showed anti-tumor activity as single agent, the efficacy of perifosine in patients with other solid malignancies was rather modest. In medulloblastoma and glioblastoma

cells perifosine was recently shown to inhibit cell growth by different independent preclinical studies [190-192]. In medulloblastoma cell lines, perifosine treatment led to suppression of AKT phosphorylation in a time-dependent and dose-dependent manner, ultimately resulting in cell death [191]. The combination of perifosine with sublethal doses of etoposide or irradiation showed additive effects in medulloblastoma cells. Perifosine also showed an impact on a glioblastoma xenograft model, where it substantially reduced phosphorylated AKT levels, inhibited tumor growth rate and had synergistic anti-proliferative effect in combination with temozolomide [190, 193]. Clinical studies are now recruiting glioblastoma patients for the treatment with perifosine as single agent and in combination with mTOR inhibitors, with the hope to reproduce the preclinical data (see Table **1**).

PI3K Inhibitors

Early studies investigating the molecular targeting of PI3K in human brain tumors used the first generation of pan-PI3K inhibitors wortmannin and LY-294002. It was reported that PI3K inhibitors impair cell growth, cell migration and invasiveness in malignant astrocytoma, glioblastoma and medulloblastoma cell lines [106-107, 194-197]. Furthermore, PI3K inhibition sensitized glioma cells to radiation-induced cytotoxicity and showed a synergistic effect with chemotherapeutic agents [196, 198-200]. Despite their efficacy as single agents, both LY-294002 and wortmannin are restricted to preclinical studies because of their toxic side effects, poor pharmacological properties and lack of selectivity [201-202]. However, they represent useful research tools that have improved our understanding of the role of PI3K in cancer biology and gave rise to the development of a new generation of PI3K inhibitors with better pharmacological properties and isoform-selectivity [203-205].

PWT-458 and PX-866, which are both pan-PI3K inhibitors, have emerged as potential therapeutic candidates because of their improved stabilities and reduced toxicities [206-207]. PWT-458 is a pegylated

derivate of wortmannin, which releases its active moiety 17-hydroxywortmannin, upon *in vivo* cleveage of its polyethylenglycol backbone. The pharmacological effect of PWT-458 in solid tumors was analyzed in preclinical settings [207]. Treatment of nude mouse xenografts with PWT-458 resulted in complete loss of AKT (Ser473) phosphorylation, demonstrating its efficacy in blocking the PI3K/AKT signalling pathway *in vivo*. PWT-458 showed potential anti-tumor activity as single-agent in xenograft models of U87MG glioma, non-small cell lung cancer and renal cell carcinoma. Additionally, in U87MG tumors, PWT-458 augmented the anti-tumoral efficacy of a suboptimal dose of paclitaxel. PX-866, another wortmannin derivate, showed potent inhibitory effects on the PI3K pathway in glioblastoma *in vitro* and *in vivo* [208-209]. PX-866 inhibited tumor growth in glioblastoma xenografts and increased the median survival time of the animals [208]. Combinations of PX-866 with BBR3610, a potent polynuclear platinum compound, resulted in synergistic cell growth inhibition in glioma cells *in vitro* and prolonged survival rates in an orthotopic xenograft model [209]. Because of its promising anti-tumor effects and its acceptable side effects shown in preclinical and clinical studies, PX-866 is presently entering Phase II clinical trials for the treatment of glioblastoma (see Table 2).

One of the first reports using a pharmacological approach to define specific PI3K family members critical for cell proliferation in glioma identified PI3K p110α as the functionally most important isoform [210]. A panel of 10 structurally unrelated isoform-selective p110 inhibitors was screened in six glioblastoma cell lines. Only inhibitors against PI3K p110α, PIK-90 and PI-103, induced a proliferative arrest. Despite the ability of inhibitors of PI3K p110β, p110δ and p110γ to block PI3K signalling through AKT phosphorylation, none of these compounds showed any inhibitory effects on cell growth. Remarkably, PI-103 was highly effective against glioma xenografts with no observable toxicities. The activity of PI-103 was ascribed to its ability to selectively block PI3K p110α and mTOR, suggesting that

combinational inhibition of PI3K p110α and mTOR is an effective therapy in malignant glioma. These results are consistent with a previous study that demonstrated that the cooperative blockade of PI3K and mTOR signalling impacts on autophagy in glioma cells [211]. Further work performed by Fan *et al*. showed that PI-103 significantly augmented the anti-proliferative effects of EGFR inhibition by erlotinib in *PTEN* mutant glioma cells [212]. This finding was of great importance, since previous studies reported that erlotinib was only able to block proliferation in *PTEN* wild-type, but not in *PTEN* mutant cells [155-156]. These results provide a noteworthy therapeutic rationale for targeting EGFR, PI3K p110α and mTOR in a combinational approach for the treatment of *PTEN* mutant tumors.

A subsequent study sought to identify the radiosensitizing potential of PI3K p110 isoform-selective inhibitors [213]. In further support of the importance of PI3K p110α in glioblastoma biology, only inhibition of PI3K p110α by PIK-90 and especially by PI-103 resulted in sensitization of glioblastoma cells to radiotherapy. Combinations of PI-103 with ionizing radiation led to a significant reduction of tumor growth rate in glioblastoma xenografts. PIK-108, a PI3K p110β inhibitor, failed to inhibit cell proliferation either alone or in combination with radiation, although it was able to reduce AKT phosphorylation. This study indicated that PI3K p110α plays a key role in the PI3K/AKT survival pathway, whereas PI3K p110β plays a more restricted role. Consistent with the aforementioned reports, this study supports the therapeutic potential of cooperatively targeting PI3K p110α and mTOR. Beside its radiosensitizing potential, PI-103 was demonstrated to sensitize glioblastoma cells to chemotherapy-induced cell death by the inhibition of DNA repair mediated by DNA-dependent protein kinase [214]. This recently published study showed for the first time that PI-103 chemosensitized glioblastoma stem cells and primary cultured glioblastoma cells to doxorubicin treatment, underscoring its clinical relevance. Together, these reports provide a rationale to overcome chemo- and radioresitance in glioblastoma. In

order to investigate tumor responses to PI-103 and to find possible biomarkers for target inhibition, a recent study monitored the effects of PI-103 by magnetic resonance spectroscopy (MRS). Phosphocholine levels were significantly decreased after PI-103 treatment of *PTEN* null prostate cells and *PIK3CA* mutant colon carcinoma cells, which correlated with ChoKα expression. Hereby, MRS detectable changes in lipid biosynthesis were identified as potential biomarkers for PI-103 mediated response [215].

In medulloblastoma, the PI3K isoform p110α was also shown to be the most important class I_A PI3K isoform in the control of cell growth and survival [107]. Downregulation by RNA interference, as well as pharmacological inhibition of PI3K p110α led to a decrease in cell proliferation and augmented the effects of chemotherapy in a cell line model. None of the inhibitors against the other isoforms, PI3K p110β or p110δ, showed a comparable effect. Recently, the gene encoding class I_B PI3K isoform p110γ, *PIK3CG*, has been found to be overexpressed in primary medulloblastoma tumor samples and cell lines. Furthermore, this isoform has been identified to contribute to cisplatin resistance, emerging as a novel target for combinatorial treatments in medulloblastoma [216]. *In vivo* studies in medulloblastoma xenografts showed that treatment with OSU03012 (PDK1 inhibitor) reduced tumor size and augmented the anti-tumor effects of CCI-779 (mTOR inhibitor) [217]. These findings suggest that dual-targeting of the PI3K/mTOR axis could be a more promising approach than single agent treatment in medulloblastoma, as has been indicated previously in glioblastoma.

It is not surprising that the dual targeting of PI3K and mTOR shows enhanced effects compared to single-agent treatment. As mentioned before, inhibitors of mTORC1 cause loss of feedback inhibition of PI3K activation in certain cell types, resulting in drug resistance. Consequently, inhibiting PI3K simultaneously with mTOR will block this feedback activation of PI3K and will therefore efficiently attenuate the PI3K/AKT/mTOR signalling cascade. In support of this notion,

dual PI3K-mTOR inhibitors are active site ATP-competitors, which target structurally related kinase domains of both PI3K and mTOR (reviewed in [179]). As previously discussed for active site mTOR inhibitors, this class of drugs demonstrated a more efficient inhibition of cell growth than rapamycin because of its ability to block the formation of both mTOR complexes, mTORC1 and mTORC2 [158].

The success achieved by dual targeting of PI3K and mTOR to inhibit tumor growth has generated great excitement for the production of new inhibitors. PKI-402, a potent dual inhibitor that targets class I PI3K, including *PIK3CA* mutants, and mTOR, showed impressive results in preclinical settings [218]. It showed anti-proliferative effects in many cell lines from breast, pancreas, non-small lung cancer and brain tumors. *In vivo*, PKI-402 reduced tumor volume in breast (MDA-MB-361) xenografts and inhibited tumor growth in glioma (U87MG) and lung (A549) xenograft models. Importantly, PKI-402 showed minimal effects on AKT phosphorylation in normal tissue, offering a therapeutic advantage.

In terms of a clinical perspective, a novel orally available dual PI3K-mTOR inhibitor with favorable pharmaceutical properties was developed by Novartis [219-220]. The imidazoquinazoline NVP-BEZ235 selectively inhibits class I PI3Ks, with higher affinity for p110α, p110δ and p110γ and mTOR. Several studies investigated the anti-tumor potential of NVP-BEZ235 in a wide range of human tumor cell lines and xenografts, including multiple myeloma, sarcoma, pancreatic cancer, breast cancer and prostate cancer [179, 218, 221-224]. NVP-BEZ235 exhibited a broad anti-tumor activity and was well tolerated when administrated orally in *in vivo* studies. In addition, NVP-BEZ235 was shown to synergize with chemotherapeutic agents, such as doxorubicin. The potential therapeutic advantage of NVP-BEZ235 is further emphasized by the fact that it also has antitumor effects in cells bearing *PIK3CA* mutations, *PIK3R1* mutations, *HER2* amplifications and *PTEN* loss [179, 221, 224]. In brain tumors, NVP-BEZ235 also demonstrated anti-tumor potential. Preclinical studies

showed that NVP-BEZ235 inhibits tumor growth in *PTEN* null glioblastoma xenografts as a single agent [220]. The results of the combination of NVP-BEZ235 with temozolomide were even more impressive, leading to regression of the tumors. Further studies on the mechanisms of action showed that NVP-BEZ235 suppressed glioma cell proliferation by efficiently inhibiting the activity of AKT and S6K, as well as the phosphorylation of ribosomal protein S6 and 4E-BP1 [225]. NVP-BEZ235 induced G1 cell cycle arrest and autophagy in glioma cells. Moreover, this study showed that NVP-BEZ235 impacts on angiogenesis, since the expression of vascular endothelial growth factor was significantly decreased upon NVP-BEZ235 treatment. Because *in vitro* experiments could be reproduced *in vivo* in animal models with similar results, this report strongly suggests NVP-BEZ235 as a candidate for the treatment of glioblastoma and other advanced cancers. Therefore, NVP-BEZ235 has entered phase I and II clinical trials in patients with malignant solid tumors. Based on the successful results obtained from targeting the PI3K/mTOR axis in preclinical studies, two further dual PI3K-mTOR inhibitors, PKI-587 and XL-765, have recently entered phase I clinical trials for the treatment of malignant gliomas. Both studies are currently recruiting patients (see Table 2). It had previously been reported from *in vitro* and *in vivo* studies that XL-765 shows favourable activity as monotherapy as well as in combination with temozolomide in a range of genetically diverse glioma xenografts [226].

In summary, inhibitors of the PI3K/AKT signalling pathway were shown to impair typical brain tumor cell properties, such as extensive cell proliferation, resistance to apoptosis, cell migration and invasiveness. It remains to be elucidated which kind of inhibitor is the most appropriate for a given cancer type. Identification of biomarkers to predict drug sensitivity would substantially facilitate the choice of a specific therapy to successfully treat defined groups of patients. For instance, tumors that are sensitive to EGFR inhibitors are *PTEN* wild type [155]. Those who present loss-of-function in *PTEN* display

resistance to EGFR inhibitors, because PI3K/AKT signalling is uncoupled from EGFR control [212]. For the latter type of tumors dual PI3K-mTOR inhibitors might be an alternative, as single agents or in combination with EGFR inhibitors. In cancers with *PIK3CA* mutations, isoform-specific inhibitors against PI3K p110α might be more effective. However, it cannot be ruled out that the non-targeted PI3K isoforms will compensate the lack of activity of the inhibited isoform. It is not clear yet whether non-selective pan-inhibitors offer better clinical benefits, because they may show additional toxicities due to broad inhibition of all isoforms. Moreover, it is plausible that single-agent PI3K/AKT pathway inhibitors have limited efficacy, because of the presence of multiple genetic alterations and feedback mechanisms found in cancer cells. It is therefore not surprising that combinatorial approaches may result in more promising results. An improved understanding of the complexity of the signalling networks inside tumor cells and their mechanisms of adaptation to compensate the effects caused by different targeted therapies will hopefully guide the development of new strategies for the treatment of brain tumors in the future.

FINAL CONSIDERATIONS AND PERSPECTIVES

Despite aggressive treatment including surgical resection, followed by chemotherapy and/or radiotherapy, the survival rates of patients with grade IV brain tumors is still very low, e.g. one year for glioblastoma patients. Additionally, this intensive treatment can cause severe side effects. Components of the pro-survival PI3K/AKT signalling pathway have been shown to be commonly altered in these tumors and seem to play an important role in the pathogenesis of malignant brain tumors. Inhibitors specific for single components of the PI3K/AKT pathway are able to affect brain tumor cells *in vitro* and *in vivo* by reducing cell proliferation, cell migration and invasion properties. Further, these inhibitors represent a useful tool in discovering the molecular biology

of brain tumors. In clinical trials, however, single inhibitor therapy has not shown highly convincing results so far. This can at least in part be explained by the fact that *in vitro* and *in vivo* models can rarely represent the vast genetic heterogeneity of tumors occurring in patients. Due to this wide spectrum of mutations in tumors with the same origin, it is not surprising that patients respond differently to a given treatment. For instance, a recent study has characterized the genetic features of advanced tumors and shown that tumors bearing *PIK3CA* mutations but not mutations in *KRAS* or *BRAF* respond to therapy targeting the PI3K/AKT signalling pathway. Patients with tumors bearing mutations in *BRAF* and *KRAS* did not respond to PI3K/AKT targeted therapy, indicating the presence of resistance mechanisms against this treatment approach [143]. Sequencing cancer genome studies will therefore be helpful to better characterize the average number of driving mutations involved in tumor formation and to gain further insights into the complex ability of tumor cells to develop resistance mechanisms to different treatment strategies. For example, resistance to radiotherapy and chemotherapy is a very common problem in glioblastoma. Additionally, resistance to protein kinase inhibitors most frequently involves mutations in gatekeeper residue of the kinase, which controls access to the affinity pockets in which most of the inhibitors bind. The multiple mechanisms leading to acquired resistance are very complex and point out the need for further studies to discover mechanistic insights and feedback loops leading to drug resistance. It has generally been observed in the clinic that multi-targeting therapies have a more potent effect than single-agent therapies and thus probably represent the more promising approach for satisfying patient responses. These therapies can involve different strategies: targeting multiple components in the same pro-survival signalling pathway, targeting multiple components in different signalling pathways or combining specific inhibitors with other treatment strategies like chemotherapy (inducing DNA damage or abolishing DNA repair mechanisms) and radiotherapy (inducing DNA damage). However, it remains challenging to predict which combinations are the most accurate for the

treatment of certain subtypes of cancer. Further studies will lead to deeper insight into the exact molecular and cellular mechanisms of malignant brain tumors and will help to develop future treatment modalities, including the discovery of potential diagnostic markers and the achievement of personalized medicine.

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ABBREVIATIONS

4E-BP1 = Eukaryotic translation initiation factor 4E-binding protein 1

BAD = BCL2-associated death promoter

EGF = Epidermal growth factor

EGFR = Epidermal growth factor receptor

FKHR = Forkhead box O1

GBM = Glioblastoma multiforme

GSK-3β = Glycogen synthase kinase 3 beta

IGF-1R = Insulin-like growth factor 1 receptor

IRS1 = Insulin receptor substrate 1

IRS2 = Insulin receptor substrate 2

iSH2 = Inter-Src homology 2

MGMT = O^6-methylguanine-DNA methyltransferase

MRS = magnetic resonance spectroscopy

mTOR = Mammalian target of rapamycin

mTORC1 = Mammalian target of rapamycin complex 1, mTOR complex with raptor

mTORC2 = Mammalian target of rapamycin complex 2, mTOR complex with rictor

nSH2 = N-terminal Src homology 2

PDGFR = Platelet-derived growth factor receptor

PDK1 = 3-phosphoinositide-dependent protein kinase 1

PI3K = Phosphoinositide 3-kinase, phosphatidylinositol 3-kinase, PI 3-kinase

PI = Phosphatidylinositol

PIP = Phosphatidylinositol-5-monophosphate

PIP2 = Phosphatidylinositol-4,5-bisphosphate

PIP3 = Phosphatidylinositol-3,4,5-trisphosphate

PTEN = Phosphatase and tensin homologue deleted on chromosome 10

RTK = Receptor tyrosine kinase

S6K = p70 ribosomal S6 kinase; ribosomal protein S6 kinase, 70kDa, polypeptide 1

SHH = Sonic hedgehog

TCGA = The Cancer Genome Atlas

TSC1 = Tuberous sclerosis complex 1

TSC2 = Tuberous sclerosis complex 2

VEGFR = Vascular endothelial growth factor receptor

Vsp34 = Vacuolar protein-orting defective 34

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